Defining the T Cell and Innate Lymphoid Cell Signature in Childhood Arthritis

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Declaration

I, Hannah R G Lom, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

Pathogenesis of juvenile idiopathic arthritis (JIA) is linked to the IL-23/IL-17 axis. This is of particular interest as new biological drugs targeting this pathway have been developed and may prove a useful treatment. Increased IL-17+CD4+ T cells have been reported in the joints of patients with JIA, but less is known about IL-17 production from other cell types. Innate lymphoid cells (ILC) bridge innate and adaptive immune systems and are sub-divided into 3 types (1,2 and 3), which mirror CD4+T- cell subsets. This thesis aimed to investigate T cell and ILC contribution to the disease phenotype in the joints of patients with JIA.

Using a combination of flow cytometry techniques along with multiplex cytokine analysis, qPCR and culture assays this study has assessed the immune phenotype of patients with JIA. Significant enrichments of IL-17A+ CD4+, CD8 and γδ T cells were observed in the synovial fluid of patients compared to blood, which correlated with disease severity. In parallel, a significant difference was seen between relative proportions and functionality of ILC subpopulations in the synovial fluid of patients compared to blood. IL17+ILC3 and IL-22+ILC3 were significantly enriched at the inflamed site. These two ILC3 populations were negatively correlated suggesting interplay between the two groups. Additionally, IL-17+ILC3 were associated with more severe disease. Further exploration of immune cells found a significant enrichment of myeloid DC (mDC), which strongly correlated with IL-22+ILC3 at the inflamed site. It was shown that synovial mDC produce retinoic acid which may contribute to skewing of ILC populations.

These data suggest a strong IL-17A signature in JIA patients, which extends beyond the T cell compartment. Therefore, therapies targeting the IL-17/IL-23 axis could also attenuate ILC populations within the inflamed joint. Finally, these data suggest that IL22+ILC3 populations are induced by mDC and may be protective in disease.

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Abbreviations

AHR	Aryl hydrocarbon receptor
Ab	Antibody
aHC	Adult healthy control
APC	Allophycocyanin
APCs	Antigen presenting cells
AS	Ankylosing spondylitis
ATRA	All-trans retinoic acid
BV	Brilliant violet
CCL	CC chemokine ligand
CCR	CC chemokine receptor
CD	Cluster of differentiation
cHC	Child healthy control
cKIT	Stem cell growth factor receptor
CRTH2	Prostaglandin D2 receptor 2
CTL	Cytotoxic T lymphocyte
CTLA4	Cytotoxic T lymphocyte antigen 4
CXCL	CXC chemokine ligand
CXCR	CXC chemokine receptor
Cy	Cyanine
D	Diversity segment encoding TCR chain
DAPI	4',6-diamidino-2-phenylindole
DC	Dendritic cells
DMSO	Dimethylsulfoxide
DMSO	Dimethylsulphoxide
EAE	Experimental autoimmune encephalomyelitis
EDTA	Ethylenediaminetetraacetic acid
EOMES	Eomesodermin
ERA	Enthesitis-related arthritis
ESR	Erythrocyte sedimentation rate
FACS	Fluorescent activated cell sorting

FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
FOXP3	Forkhead box P3
g	Gravity
GATA3	GATA binding protein 3
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GOSH	Great Ormond Street Hospital
HLA	Human leukocyte antigen
IBD	Inflammatory bowel disease
IFN	Interferon
IL	Interleukin
ILAR	International league of associations for rheumatology
ILC	Innate lymphoid cells
J	Joining segment encoding TCR chain
JIA	Juvenile Idiopathic Arthritis
KIR	Killer-cell immunoglobulin-like receptor
LPS	Lipopolysaccharide
MAMPs	Microbe-associated molecular patterns
mDC	Myeloid dendritic cells
MFI	Mean fluorescence intensity
MHCI	Major histocompatibility complex I
MHCII	Major histocompatibility complex II
MS	Multiple sclerosis
MTX	Methotrexate
NCR	Natural cytotoxicity receptor
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural killer
NKp44	Natural cytotoxicity receptor 44
NKR-P1A	Natural killer receptor protein 1A
oligo	Oligoarticular
p	Probability
P/S/glu	Penicillin/streptomycin/glutamine
PAMPs	Pathogen-associated molecular patterns

PB	Peripheral blood
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pDC	Plasmacytoid DC
PE	R-phycoerythrin
PerCP	Peridinin chlorophyll protein
PMA	Phorbol myristate acetate
poly	Polyarticular
PRR	Pattern recognition receptors
PsA	Psoriatic
RA	Retinoic acid
RALDH	Retinaldehyde dehydrogenase
RAR	Retinoic acid receptor
RF	Rheumatoid factor
rh	Recombinant human
RORC	Retinoic acid-related orphan nuclear hormone receptor C
SF	Synovial fluid
SFMC	Synovial fluid mononuclear cells
SNP	Single nucleotide polymorphisms
SpA	Spondyloarthritis
STAT	Signal transducer and activation of transcription
T conv	Conventional T cells
T-bet	T-box expressed in T cells
TCR	T cell receptor
TE	Tris-EDTA
TGF	Transforming growth factor
Th	T helper cell
TLR	Toll-like receptor
TNF	Tumour necrosis factor
Treg	Regulatory T cells
TSDR	Treg-specific demethylated region
TSLP	Thymic stromal lymphopoietin

UCLH	University College Hospital London
V	Variable segment encoding TCR chain

Table of Contents

List of Tables

List of Figures

1 : Introduction

1.1 Juvenile Idiopathic Arthritis

1.1.1 Definition of Juvenile Idiopathic Arthritis (JIA)

Juvenile idiopathic arthritis (JIA) is a term for a group of conditions, which together represent the most common rheumatic disease of childhood (Ravelli and Martini, 2007). JIA is defined by arthritis of unknown cause, etiology in one or more joints, which onsets before the age of 16 and persists for at least 6 weeks. JIA covers a heterogeneous group of diseases, which can be divided into distinct subsets based on clinical manifestations Table 1.1 denotes the classification criteria and clinical presentations for JIA subtypes. (Petty et al., 2004)

1.1.1.1 Classification of Enthesitis Related Arthritis (ERA JIA)

While many subtypes of JIA have been well investigated, the pathogenesis of ERA JIA is still illusive. The initial focus of this thesis will be to define pathogenic immune responses in the ERA JIA subtype. ERA JIA occurs more frequently in males than in females with a ratio of 6-7:1, with onset most commonly in adolescence. ERA JIA has a close genetic association with the HLA-B27 allele. The strength of this association increases with age of onset (Murray et al., 1999). ERA JIA can be broadly divided into two subtypes with differential prognosis according to HLA-B27 status and age of onset (Berntson et al., 2008). Early onset ERA JIA tends to present with less severe, peripheral arthritis. This type of ERA JIA has a lower rate of the HLA-B27 allele, and usually responds well to treatment. More severe ERA JIA tends to onset during adolescence and has higher incidence of HLA-B27. This phenotype has a poor prognosis frequently resulting in the involvement of axial and sacroiliac joints. This second phenotype is more similar to the well-recognised adult condition, known as ankylosing spondylitis (AS) or spondyloarthropathy (SpA) (Fisher et al., 2011, Corinne Fisher, 2012).

Table 1.1 Classification criteria and clinical presentations for JIA subtypes

Category	Definition	Exclusions
Systemic Arthritis (Sys JIA)	Arthritis in one or more joints with or preceded by fever of at least 2 weeks duration that is documented daily ("quotidian") for at least 3 days, and accompanied by one or more of the following: 1. Evanescent (nonfixed) erythematous rash 2. Generalised lymph node enlargement 3. Hepatomegaly and/or splenomegaly 4. Serositis	A. Psoriasis or a history of psoriasis in the patient or first degree relative B. Arthritis in an HLA-B27 positive male beginning after the 6th birthday C. Ankylosing spondylitis, enthesitis related arthritis, sacroilitis with inflammatory bowel disease, Reiter's syndrome, or acute anterior uveitis, or history of one of these disorders in in a first degree relative D. The presence of IgM rheumatoid factor on at least 2 occasions at least 3 months apart
Oligoarticular Arthritis (Oligo JIA)	Arthritis affecting 1-4 joints during the first 6 months of disease. Two subcategories are recognized: 1. Persistent oligoarthritis (P-Oligo): affecting not more than 4 joints throughout the disease course 2. Entended oligoarthritis (E-Oligo): Affecting a total of more than 4 joints after the first 6 months of disease	A, B, C, D above plus, E. The presence of systemic JIA in the patient
Polyarticular Arthritis (Poly JIA)	Arthritis affecting 5 or more joints during the first 6 months of disease	A, B, C, E
Psoriatic Arthritis (PsA JIA)	Arthritis and psoriasis, or arthritis and at least 2 of the following: 1. Dactylitis 2. Nail pitting or onycholysis 3. Psoriasis in a first-degree relative	B, C, D, E
Enthesitis Related Arthritis (ERA JIA)	Arthritis and enthesitis, or arthritis or enthesitis with at least 2 of the following: 1. The presence of or a history of sacroiliac joint tenderness and/or inflammatory lumbosacral pain 2. The presence of HLA-B27 antigen 3. Onset of arthritis in a male over 6 years of age 4. Acute (symptomatic) anterior uveitis 5. History of ankylosing spondylitis, enthesitis related arthritis, sacroilitis with inflammatory bowel disease, Reiter's syndrome, or acute anterior uveitis in a first degree relative	A, D, E
Undifferentiated	Arthritis that fulfills criteria in no category or in 2 or more of the above	n/a

1.1.2 Pathogenesis of JIA

1.1.2.1 Genetic associations

There is some evidence for a genetic contribution to JIA pathogenesis, which is highlighted by familial aggregation of the disease (Prahalad et al., 2000, Prahalad et al., 2004, Prahalad et al., 2010). The strongest genetic factors affecting JIA are associated with alleles within the MHC region, and the allele associations differ according to JIA subtype(Edmonds et al., 1974, Thomson et al., 2002). Most of the JIA subtypes defined above are primarily associated with MHC class II alleles. In particular, HLA-DR alleles have been heavily implicated(Thomson et al., 2002, Zeggini et al., 2002, Hinks et al., 2016). Of these, HLA-DRB1*, HLA-DRB1*1101 and HLA-DRB1*1301 are associated with oligo-JIA; HLA-DRB1*801 and HLA-DRB1*0401 are linked to poly-JIA(Donn et al., 1995). Indeed these findings have been confirmed in immunoCHiP studies where the primary genetic association was *HLA-DRB1,* and in particular *HLA-DRB1* alleles which share a histidine amino acid at position 13, which was shown to contribute 50% of HLA-associated variance (Hinks et al., 2016). Within the MHC class I loci, HLA-B27 has been shown to be strongly associated with ERA JIA (Hinks et al., 2016). The putative roles for HLA-B27 in ERA JIA pathogenesis will be discussed further in section 1.1.2.3.1.

Outside of the MHC loci, several polymorphisms in genes associated with cytokines have also been described. A significant link between intronic +851 TNFα SNP and oligo-JIA has been reported (Zeggini et al., 2002). A putative role for the IL-23/IL-17 axis has been described in JIA, and a rs1004819 polymorphism in the IL-23R gene has been shown to be protective in poly-JIA (Emami et al., 2016). Additionally, SNPs in the IL-6 gene have been reported (Crawley et al., 1999)and are associated with systemic-JIA. Specifically, the 174G/C polymorphism in the IL-6 gene is associated with systemic JIA susceptibility (Fishman et al., 1998, Ogilvie et al., 2003). Mutations in the IL-10 gene have also been described in oligo-JIA patients, which result in impaired IL-10 production (Crawley et al., 1999). Finally, polymorphisms in genes

associated with T cell function including *PTPN22*, which is involved in T cell activation, have also been described and support a role for T cells in disease pathogenesis(Hinks et al., 2005). These targeted studies have been subsequently validated using the immunoCHiP platform where 14 non-HLA loci, including *PTPN22, PTPN2, IL-2RA and STAT4,* were identified as significantly associated with JIA susceptibility at genome wide level (Hinks et al., 2013).

1.1.2.2 Immunopathology of JIA

During inflammatory arthritis, including JIA, the synovia of patients are infiltrated by immune cells and become engorged. Many studies have been conducted investigating the immune cells at the site of inflammation in order to determine the mechanism of pathogenesis. These studies have focused predominantly on oligo-JIA and poly-JIA subtypes, which will be discussed in this section. The immune pathogenesis of ERA JIA will be considered independently in section 1.1.2.3.

Several members of the innate system have been identified as abnormal in JIA. High levels of the S100 protein myeloid reactive protein (MRP)8/14 have been detected in the joints of patients with active arthritis, which alludes to monocyte and neutrophil involvement in disease pathogenesis (Frosch et al., 2009). Indeed, synovial monocytes, though similar in absolute number, are functionally different in the joint, where they are highly activated (Hunter et al., 2010). In addition to MRP8/14, synovial monocytes from JIA patients have been shown to secrete numerous inflammatory cytokines including IL-6, IL-1β and TNFα as well as IFNγ (Saxena et al., 2005, Hunter et al., 2010) .Furthermore, the hypoxic conditions of the JIA synovia has been shown to induce CCL20 secretion from monocytes, a chemokine known to be significant for the recruitment of immune cells to the site of inflammation (Bosco et al., 2008). Several gene families linked to IL-8 and IFNγ have been identified as differentially expressed in JIA patients compared to healthy control blood, supporting a role for neutrophils in disease pathogenesis. Expression of these genes remained abnormal even during disease remission (Jarvis et al., 2006). Although relatively poorly

investigated, myeloid dendritic cells (mDC) have been identified in increased frequency in the inflamed joints of JIA patients, where they mediate disease via the secretion of receptor activator of NFκB (RANK)(Varsani et al., 2003, Smolewska et al., 2008, Tabarkiewicz et al., 2011). Binding of the ligand (RANKL) to RANK results in osteoclast formation and bone degradation. Interestingly, hypoxia is shown to induce triggering receptor expressed on myeloid cells 1 (TREM-1) expression on monocyte derived DC, and the induction of proinflammatory signature. This finding has been confirmed in synovial mDC from JIA patients supporting a highly inflammatory and potentially pathogenic role for these cells at the inflamed site(Bosco et al., 2011).

The strong HLA-associations highlight the significance of T cells in JIA, given the central role of MHC molecules in antigen presentation to T cells. Due to the previously defined association of oligo-JIA and poly-JIA to MHC class II alleles, it is unsurprising that research to date has focused on CD4 T cell populations. Indeed, CD3 T cells are usually the most abundant cell type within the synovial fluid mononuclear cells, and are typically highly activated memory cells (Silverman et al., 1993, Wedderburn et al., 2000a, Chiesa et al., 2004). Investigation into T cell subtypes at the inflamed site has identified Th1 and Th17 cell expansions at the inflamed site, which are likely to have been preferentially recruited via the respective expression of CCR5, CXCR3 and CCR6(Wedderburn et al., 2000a, Nistala et al., 2008a). Although, IFNγ blockade does not yield beneficial outcomes in murine models of inflammatory arthritis, it is possible that Th1 cells contribute to pathogenesis via the production of TNFα, which is routinely, and successfully used for treatment of JIA(Lovell et al., 2000, Brasted et al., 2005). Highlighting a role for IL-17 in JIA pathogenesis, Th17 cells and IL-17 are both increased in the synovial fluid and are associated with worse disease outcomes (Nistala et al., 2008a, Agarwal et al., 2008b). Significant Th17 plasticity has been reported at the inflamed site in JIA, where synovial IL-12 is shown to drive the conversion of Th17 cells towards IFNγ and GM-CSF producing phenotypes (de Jager et al., 2007, Nistala et al., 2010a, Piper et al., 2014a). Interestingly, one study investigating the clonality of synovial T cells in JIA observed significant oligoclonality in T cell subsets, which varied according to JIA subtype. Significant CD4 T cell clonality was seen in

oligo-JIA, supporting the hypothesis that disease pathogenesis may be, at least in part, antigen driven (Wedderburn et al., 2001). Although CD8 cells are not frequently studied in oligo- and poly-JIA subtypes, an expansion of CD8 T cells has been reported, and an inverted CD4:CD8 ratio is associated with more severe disease(Hunter et al., 2010).

In addition to effector T cells, regulatory T cells (Treg) are also enriched within the synovial fluid of JIA patients compared to the patients' blood, although lower frequencies are reported in more severe subtypes of the disease (de Kleer et al., 2004, Nistala et al., 2008a). There is still some debate over the functionality of Treg at the inflamed site. In vitro studies have demonstrated equal suppressive capacity of synovial Treg compared to Treg isolated from peripheral blood(Wehrens et al., 2011). However, neither synovial nor peripheral blood Treg are able to inhibit proliferation of effector T cells from the synovial fluid (Wehrens et al., 2011). More recent studies in JIA have identified a population of Treg that express high levels of CD25 but reduced expression of FOXP3. Further analysis demonstrated robust suppressive activity and TSDR demethylation in these $FOXP3^{low}$ cells(Bending et al., 2014). Additionally, there was significant TCR repertoire overlap between FOXP3 positive and FOXP3 negative CD25 expressing populations(Bending et al., 2015). Together these studies suggest uncoupling of FOXP3 with the TSDR, and functional FOXP3 negative Treg cells may exist in inflamed sites.

In contrast to their traditional role in controlling aberrant inflammation, there is growing evidence that Treg at the inflamed site may in fact exacerbate inflammation. A population of Treg with effector functions has recently been identified at the inflamed site, characterised by CD161 expression(Pesenacker et al., 2013, Duurland et al., 2017a). Additionally, abnormal Treg responses and significant repertoire sharing across patients has been reported in JIA, suggesting that antigen recognition by Treg could also play a role in disease pathogenesis(Henderson et al., 2016).

1.1.2.3 Pathogenesis of ERA JIA

1.1.2.3.1 HLA-B27 mediated immunopathology of ERA JIA

As previously alluded to, the MHC Class I HLA-B27 allele is the strongest genetic factor associated with ERA JIA and presence of this allele is linked to more severe disease and axial joint involvement (Fisher et al., 2011, Corinne Fisher, 2012). The exact role of HLA-B27 in disease pathogenesis is still unclear, although several hypotheses have been proposed and investigated in adult spondyloarthropathies and animal models of disease.

One putative mechanism for HLA-B27-mediated ERA JIA is the presentation of arthritogenic peptides to CD8 T cells via MHC class I. ERA is also associated with polymorphisms in the gene for endoplasmic reticulum aminopeptidase (ERAP1), an enzyme involved in the optimisation of peptides for MHC presentation, which gives weight to this hypothesis(Saric et al., 2002, Hinks et al., 2011). However, HLA-B27 transgenic rat models of disease have discredited this theory, as rats continued to develop disease in the absence of CD8 T cells(May et al., 2003, Taurog et al., 2009).

A second hypothesis for HLA-B27 focuses on HLA-B27 protein misfolding within the endoplasmic reticulum (ER) (Mear et al., 1999). This misfolding has been shown in rat models and myeloid derived DC to invoke ER stress, which results in the up regulation of IL-23 in response to lipopolysaccharides (LPS)(DeLay et al., 2009, Goodall et al., 2010). This enhanced IL-23 production could in part explain the expanded Th17 populations reported in the inflamed site of SpA patients, as IL-23 is known to be significant for Th17 differentiation and function (Shen et al., 2009, Jansen et al., 2015, Bowness et al., 2011). However, again, doubt has now been cast over this putative mechanism of pathogenesis for HLA-B27. A recent study failed to identify evidence of deregulated unfolded protein response–associated genes in the inflamed gut in adult AS, but instead implicated autophagy (Ciccia et al., 2014a).

The final hypothesis proposes a direct role for HLA-B27 in disease pathogenesis, and more specifically in directing Th17-type immune responses. Free HLA-B27 heavy chains have been shown to form disulphide-bonded homodimers, which are expressed both intracellularly and/or on the cell surface (Allen et al., 1999, Kollnberger et al., 2004, Colbert et al., 2014). Although expression of these homodimers have not been examined in ERA JIA, their expression has been observed on PBMC in adult AS(Tsai et al., 2002, Raine et al., 2006, Payeli et al., 2012). In addition, it has been shown that these heavy chain homodimers can be directly recognised by killer-immunoglobulin-like receptors (KIRs) expressed on NK cells and CD4 T cells (Kollnberger et al., 2002, Kollnberger et al., 2007, Cauli et al., 2013). Subsequent investigations demonstrated increased expression of KIR3DL2 on CD4 T cells from PBMC taken from HLA-B27-positive healthy controls and AS patients. These KIR3DL2 expressing CD4+T cells produced IL-17 following stimulation, and expressed the Th17 master transcription factor RORC(Bowness et al., 2011, Ridley et al., 2016). Interestingly, silencing of ERAP1 has been shown to down regulate expression of HLA-B27 heavy chain homodimers and suppress Th17 responses in vitro (Chen et al., 2016).

1.1.2.3.2 Immune pathology of ERA JIA

Investigation of ERA JIA pathogenesis to date has been limited, and has focused predominantly on innate immune cell involvement. Microarray analysis of unsorted PBMC and SFMC from ERA JIA patients has identified overexpression of genes related to antigen presentation and scavenger function in cells from the joint compared to ERA JIA blood (Myles et al., 2012). Other studies have shown that monocyte-derived pro- inflammatory molecules such as the S100 protein MRP8/14 are elevated in the synovial fluid of ERA JIA patients and the expression of toll like receptors (TLR) 2 and 4 is increased on peripheral blood and synovial fluid monocytes in ERA JIA patients (Saxena et al., 2005, Myles and Aggarwal, 2011, Rahman et al., 2014). To date there has been no analyses of the recently defined innate lymphoid cells (ILC), their phenotype or relative abundance in the joints of patients with ERA JIA: although

given their potential for inflammatory cytokine production this area certainly warrants investigation.

Very few studies have been conducted investigating T cell involvement in ERA JIA pathogenesis. One study analysed T cell receptor clonality in ERA JIA compared to oligo-JIA and found that the oligoclonality, was more marked in CD8 cells in ERA JIA patients. This is intriguing given the link to a class I MHC allele, HLA- B27 (Wedderburn et al., 2001). An initial study has suggested Th17 cell enrichment in the synovial fluid mononuclear cells of ERA JIA patients in an Indian cohort (Mahendra et al., 2009). More recent reports have also demonstrated expanded IL-17 producing NK and γδ T cell populations within the peripheral blood from ERA JIA patients(Gaur et al., 2015).

The involvement of IL-17-producing cells in ERA JIA could have important implications for the treatment of this subtype of JIA as several new therapies targeting the IL-17/IL-23 axis have been developed. These treatments include blockade of IL-17 by drugs such as Secukinumab, of IL-23 (e.g. by the drug Guselkumab), and of the common p40 subunit, which is shared by IL-12 and IL-23, which is blocked by drugs such as Ustekinumab.

1.1.2.3.3 Pathogenesis of adult spondyloarthropathies

Similarly to ERA JIA, adult AS is characterized by enthesitis and axial joint involvement and shares strong genetic associations with the HLA-B27 allele and ERAP1(Schlosstein et al., 1973, Newport et al., 2007, Chen et al., 2014b). In fact, ERA JIA is, by some, considered to be young onset AS. The pathogenesis of AS has been extensively examined and may provide clues to direct investigation of ERA JIA. Over 40 AS-associated genes have been identified in genome wide association studies (GWAS)(Cortes et al., 2013). Of note, AS has been linked to the IL-23/IL-17 axis in both genetic and immune analyses. Polymorphisms in genes associated with IL-23 and IL-6 (two cytokines known to be important for the differentiation of Th17 cells from naïve CD4 T cells) receptor signalling have been shown to be associated with disease

susceptibility in AS (Newport et al., 2007, Zhou et al., 2007, McGeachy et al., 2009, Cortes et al., 2013)

Translating results from genome wide association studies (GWAS) to immune mechanisms, the IL-23/IL-17 axis has been further implicated in pathogenesis in both human AS and murine models. There is significant evidence of an expanded Th17 population in the peripheral blood from AS patients(Shen et al., 2009, Bowness et al., 2011, Jansen et al., 2015). Additionally, IL-17+ γδ T cells, CD8 MAIT cells, and ILC have also been identified in the peripheral blood, synovial fluid and ilium from patients(Kenna et al., 2012, Ciccia et al., 2015, Gracey et al., 2016). Additionally, murine models of AS suggest entheseal inflammation in may be attributed to with IL-23 activated tissue resident T cells, although this is yet to be confirmed in human disease(Sherlock et al., 2012).

1.1.3 Current strategies for the treatment of JIA

The aim of treatment of JIA is to achieve complete control of the disease whilst preventing long-term side effects of therapy (Ravelli and Martini, 2007). The Juvenile Arthritis Disease Activity Score (JADAS) is a clinical tool used for assessing disease severity in JIA patients and takes into consideration biological measures of disease (active and tender joint counts and erythrocyte sedimentation rate (ESR), as well as more the more holistic physician and parent's global assessments (Consolaro et al., 2009, Wu et al., 2016).

Treatment for JIA varies on a patient-by-patient basis, and according to JIA subtype, however similar approaches are often implemented. In addition to physiotherapy and occupational therapy, a plethora of drugs are deployed. Non-steroidal anti-inflammatory drugs (NSAIDs), most commonly Naproxen, are usually the first line of treatment. Patients often also receive intra-articular injections of corticosteroid. During this procedure, corticosteroid is injected into the inflamed joints, and excess synovial fluid is aspirated. If these first line treatments fail, patients may enter into more aggressive treatment programs, such as systemic corticosteroids or disease modifying anti-rheumatic drugs

(DMARDs), such as methotrexate (MTX) (Ruperto et al., 2004). These treatments are effective in approximately 50% of cases. Unresponsive patients may then be considered for biological treatments. The most common of these are anti-TNFα agents, Etanercept (recombinant soluble TNF-R –Ig fusion protein) or one of the monoclonal antibodies to TNFα Infliximab or Adalimumab, which are generally well tolerated, and result in significant improvements in many patients(Lovell et al., 2006). However, systemic JIA-patients do not generally respond well to these agents, and anti-IL-6 receptor, Tocilizumab; or recombinant IL-1 receptor antagonist, Anakinra, are more effective for the treatment of this subtype(Klein and Horneff, 2009).

More recently, biological treatments targeting the IL-23/IL-17 axis have been developed and appear effective for the treatment of adult arthropathies. Secukinumab, which targets IL-17A, and Ustekinumab, which targets the p40-shared subunit between IL-23 and IL-12, are shown to be effective for the treatment of adult ankylosing spondylistis in clinical trials(Baeten et al., 2013, Poddubnyy et al., 2014). These treatments are yet to be trialled in JIA, although a clinical trail for Secukinumab in JIA is currently being established.

The response to treatment in JIA is, in most studies, measured according to the American College of Rheumatology (ACR) criteria(Giannini et al., 1997). Treatment is deemed successful when patients achieve at least 70% improvement in 3 out of the 6 core outcome variables, with no more than one getting 30 % worse or more, defined as patients reaching ACR70 or above. Unfortunately, remission is only achieved in a minority of patients, and therefore improved treatment strategies are required.

1.2 The immune System

The immune system can be broadly divided into innate and adaptive arms, which together provide protection and host defence against exogenous (pathogens) and endogenous (e.g. apoptotic cells) noxious stimuli. Despite differential functions, there is significant overlap and cross talk between the innate and adaptive immune systems, both in initiating immune responses and in ensuring regulation of inflammation and maintaining immune homeostasis.

The innate immune system in conjunction with the epithelia forms the first-line of response to invading pathogens. Innate cells are short lived and act non-specifically to commensals and pathogens. Innate leukocytes such as monocytes, dendritic cells (DC) and natural killer (NK) cells are poised to respond and are activated upon recognition of preserved microbe-associated molecules (MAMPS) via pattern recognition receptors (PRR)(Medzhitov and Janeway, 2002, Janeway and Medzhitov, 2002). Following their activation these cells will lyse or phagocytose foreign and infected cells. Microbial antigens are then presented by professional antigen presenting cells (APC), via the major histocompatibility complex (MHC) I or II, to members of the adaptive immune system. In addition, innate immune cells produce a plethora of inflammatory mediators such as cytokines and chemokines that recruit other cells to the sites of inflammation and are pivotal in directing the magnitude and outcome of the adaptive immune response.

In contrast to their innate counterparts, cells of the adaptive immune system are slower to respond, are highly specific and develop memory, such that they are able to respond more quickly and with higher affinity upon second and repeat exposure to the same microbe. T- and B-lymphocytes are components and responsible for adaptive immune responses. These cells detect antigens presented by APC via interaction with their highly specific T cell and B cell receptors (TCR/BCR). Once activated, these lymphocytes respond by the production of further inflammatory mediators, by killing of infected cells and by the production of antibodies(Abbas, 2011).

1.2.1 Innate immunity

The innate immune system acts as the first line of response against infection, but in certain conditions such as in genetically susceptible individuals, innate immunity may also contribute to aberrant inflammation and autoimmunity. As innate lymphoid cells and myeloid dendritic cells were major cell-types investigated in this thesis, it is appropriate to describe their known structure and function in greater detail.

1.2.1.1 Innate Lymphoid Cells

Innate lymphoid cells (ILC) are a recently discovered population of lymphocytes. As the name suggests, ILC contribute to innate immunity. NK cells were the first ILC population to be discovered and could be considered to be analogous to cytotoxic CD8 T cells, mediating inflammation by inducing death of infected cells. Lymphoid tissue inducer cells (LTi) also fall under the ILC umbrella, and play a pivotal role in the formation and maintenance of lymphoid tissues (Mebius et al., 1997, Hazenberg and Spits, 2014a). Non-cytotoxic "helper" ILC populations, which are identified by the expression of the IL-7 receptor alpha chain (CD127) and can be broadly divided into 3 major subsets (ILC1, ILC2 and ILC3)(Spits et al., 2013). These subpopulations, in some respects mirror CD4 helper T (Th) cell subsets (Th1, Th2 and Th17/Th22), based on their cytokine and transcription factor profiles (Walker et al., 2013, Hazenberg and Spits, 2014b). T cell immunity will be discussed in more detail in section 1.2.2.1. Unlike their respective T cell counterparts, ILC lack RAG-rearranged genes for antigen receptors. Instead, ILC are acutely sensitive to their local environment and, following cytokine stimulation, become potent cytokine producers (Hoorweg et al., 2012). Since their discovery, ILC subsets have been implicated in the early stages of pathogenesis of a multitude of diseases, as well as being important mediators of repair and homeostasis. To date, very little has been reported about the role of ILC in arthritis (Hazenberg and Spits, 2014b). ILC subtypes are depicted in Figure 1.1.

1.2.1.1.1 ILC development

Investigations into the development of ILC populations are largely restricted to murine studies. In this context, it has been shown that ILC, both NK cells and non-cytotoxic ILC, originate from common lymphoid precursors (CLP). ILC subpopulation progenitors can be subdivided into alpha-lymphoid progenitors (α-LP) and early innate lymphoid progenitor (EILP). However, more specific committed helper ILC progenitors (CHILP) have been identified, which lack the potential to develop into NK cells. Subsequent helper ILC development from these progenitor cells is dependent on expression of the transcription factors thymocyte selection–associated high- mobility group box (TOX), nuclear factor interleukin 3 regulated (NILF3), T-cell factor 1 (TCF1) and nuclear repressor Id2 (Cherrier et al., 2012, Constantinides et al., 2014, Yu et al., 2014, Yang et al., 2015, Scoville et al., 2016). Non-cytotoxic ILC are dependent on IL-7 for their development and typically express the alpha chain of the IL-7 receptor (CD127). Subsequent differentiation of ILC into their respective subsets is reliant on their cytokine environment. While this part of the differentiation process is less well defined, it is likely that the same factors are involves as for late stage T cell differentiation into their effector states (Vosshenrich and Di Santo, 2013)(Hazenberg and Spits, 2014).

The process of ILC development in humans is poorly defined; understanding is currently limited to ILC1 and ILC3 subpopulations. It is known that, similar to mice, human helper ILC arise from CHILP, and the transcription factor promyelocytic leukaemia zinc finger (PLZF) can additionally be used to identify ILC1 precursors (Constantinides et al., 2015). By contrast, ILC3 may develop from RORC+ CD34+ hematopoietic stem cells under stimulation with stem cell factor (SCF), IL-7 and IL-15 (Montaldo et al., 2014). Furthermore, there is now evidence for a role of retinoic acid and ligands of the aryl hydrocarbon receptor (AhR) in promoting ILC3 development from early committed precursors(Lee et al., 2011, van de Pavert et al., 2014). Further investigation of ILC differentiation in human cells needs to be clarified; analysis of ILC repopulation after stem cell transplantation may provide useful insight.

1.2.1.1.2 Non-cytotoxic ILC subpopulations (CD127+)

CD127+ ILC comprises a heterogeneous population of cells defined by lymphoid morphology paired with the absence of rearranged antigen receptors and other surface molecules associated with previously identified lineages (T cells, B cells, monocytes, DC, stem cells, eosinophils, or mast cells). To date, ILC can be divided into 3 subsets (ILC1, ILC2 and ILC3), which share cytokine and transcription factor signatures with Th subsets (Th1, Th2, Th17 respectively). ILC are thought to be predominantly tissue resident cells, where they mediate both inflammation and tissue repair via the production of cytokines, in response to the local milieu. CD127+ ILC help maintain mucosal homeostasis by providing anti-microbial immunity (Scandella et al., 2008, Cella et al., 2009, Sonnenberg et al., 2012). It is however becoming clear that when dysregulated, ILC may also promote damage and contribute to disease pathogenesis such as in inflammatory bowel disease and arthritis (Hazenberg and Spits, 2014a).

1.2.1.1.2.1 Group 1 Innate Lymphoid Cells (ILC1)

ILC1 have been identified in several organs including the gut and the lungs and play an important role in immunity against intracellular pathogens. Murine models have identified a protective role for ILC1 against parasite infection by inducing recruitment of myeloid cells to the site of infection, and against bacterial infection, where ILC1-secreted IFNγ protects the epithelial barrier by promoting release of glycoproteins, which form a protective mucus(Klose et al., 2014). Additionally, ILC1 have been implicated in the pathogenesis of human autoimmunity and are significantly expanded in colons of Crohn's patients(Bernink et al., 2013b). In addition, ILC1-like cells have also been identified in the synovial fluid from patients with rheumatoid arthritis and ankylosing spondylitis (Dalbeth and Callan, 2002, Leijten et al., 2015, Yeremenko et al., 2015).

Two predominant populations of ILC1 have been described, which diverge according to expression of CD127 as well as differential expression of T-box expressed in T cells (Tbet) and Eomesodermin (Eomes) transcription factors. Similarly to Th1 cells, the master transcription factor of CD127+ILC1 is Tbet. There is significant functional overlap between CD127+ILC1 and NK cell populations. However CD127+ILC1 lack expression of Eomes, a transcription factor, which is therefore useful when discriminating between these two subsets. Following activation and stimulation with IL-12 and IL-18, ILC1 rapidly produce IFNγ as well as TNFα (Fuchs et al., 2013).

Interestingly, human ILC1, expressing CXCR3 and Tbet without Eomes, have been shown to express several T cell associated markers intracellularly, including CD3 (Roan et al., 2016b). Further supporting a close association between ILC1 and T cell subsets, single cell RNA-seq of ILC1 (identified as lineage- CD127+CRTH2–CD117–NKp44–), isolated from healthy human tonsils, has shown that ILC1 express transcripts for T cell associated genes encoding for CD5 and CD6 (Bjorklund et al., 2016). Unlike some other ILC subsets, ILC1 do not express MHC class II molecules and are therefore incapable of antigen presentation to T cells; their major contribution instead is *via* IFNγ mediated inflammation.

1.2.1.1.2.2 Group 2 Innate Lymphoid Cells (ILC2)

ILC2, initially named nuocytes, mirror Th2 cells, are regulated by GATA- binding factor 3 (GATA3) and can be identified by expression of the prostaglandin D2 receptor, CRTH2. Similar to Th2 cells, ILC2 are involved in the responses against extracellular pathogens(Mjosberg et al., 2011b). ILC2 are potent producers of IL-4, IL-5 and IL-13 in response to stimulation with IL-25, IL-33 and thymic stromal lymphopoietin (TSLP), which are commonly released during tissue damage and allergic reaction (Hazenberg and Spits, 2014a).

ILC2 are the most prevalent ILC subtype in healthy blood, although they are also implicated in the allergic response and protection against helminth

infections(Hazenberg and Spits, 2014a). Several studies have shown an important role for these cells in promoting airway hyper reactivity in asthmatic models, where they are major contributors to the total IL-5 and IL-13 production (Fort et al., 2001, Lund et al., 2013, Drake and Kita, 2014). In fact these studies have shown that ILC2 produce increased IL-5 and IL-13 compared to their T cell counterparts in this context, demonstrating their pivotal role in allergic responses, which has shifted the focus in this area away from T cells(Halim et al., 2012, Bartemes et al., 2012).

ILC2 are known to interact with, and direct the function of, cells of the adaptive immune system. IL-5, IL-6 and IL-13 are readily produced by ILC2 and may mediate the activation of naïve T cells, or the production of IgA responses from B cells (Halim et al., 2014, Drake et al., 2016). In addition to production of cytokines, ILC2 may also function as antigen presenting cells. A number of studies have identifies expression of MHC class II and co-stimulatory molecules, CD80 and CD86, on the surface of ILC2, suggesting that these cells may activate T cell responses via antigen presentation (Oliphant et al., 2014).

1.2.1.1.2.3 Group 3 Innate Lymphoid Cells (ILC3)

ILC3 require retinoic acid-related orphan nuclear receptor C (RORC) for their development and function, and include non-cytotoxic ILC3 as well as tissue inducer cells (LTi)(Luci et al., 2009). For the purpose of this thesis, only CD127+ILC3, non-cytotoxic, populations will be discussed. ILC3 produce Th17 type cytokines, including IL-17, IL-22 and GM-CSF, in response to stimulation with IL-23 and IL-1β. ILC3 can be further divided into 2 groups with differential cytokine and transcription factor profiles. These two distinct populations can been distinguished according to expression of natural cytotoxicity receptor (NCR) NKp44 (Spits et al., 2013).

1.2.1.1.2.3.1 NCR-ILC3

NCR-ILC3, and more specifically, HLA-DR+ NCR-ILC3 are potent producers of IL-17, and are instrumental for the containment of commensals in the gut (Sonnenberg et al., 2012, Hoorweg et al., 2012, Bjorklund et al., 2016). Additionally, mouse models show that MHC class II-expressing NCR-ILC3 mediate commensal tolerance via antigen presentation to, and killing of, commensal reactive CD4 T cells (Hepworth et al., 2013). Although this role for NCR-ILC3 has not yet been confirmed in humans, this population is decreased in the colon of paediatric IBD patients, suggesting comparability to mouse models (Hepworth et al., 2015).

Conversely, NCR-ILC3 have been described in the gut of experimental models of colitis, as well as human Crohn's patients where they are believed to contribute to disease via the production of IL-17A (Geremia et al., 2011) Perhaps unsurprisingly, in addition to ILC1, NCR-ILC3 have been identified as the predominant ILC subtype in the inflamed synovia of AS patients (Yeremenko et al., 2015, Ciccia et al., 2015).

1.2.1.1.2.3.2 NCR+ILC3

NCR+ILC3 typically produce reduced IL-17 compared to NCR-ILC3, but are instead potent producers of IL-22 (Taube et al., 2011). This ILC3 subpopulation is somewhat parallel to proposed Th22 cells in humans. NCR+ILC3 are most prevalent in the healthy gut where they are play an important role in gut homeostasis and wound healing, by regulation of IL-17 production via AHR, and by direct interaction of IL-22 with epithelial cells (Cella et al., 2009, Hepworth et al., 2013, Qiu et al., 2013). In addition, murine NCR+ILC3 have recently been shown to produce GM-CSF in a model of inflammatory bowel disease. In this context, they mediate Treg responses via the GM-CSF-dependent induction of retinoic acid from myeloid cells (Mortha et al., 2014). On the other hand NCR+ILC3 may also contribute to inflammation via the production of inflammatory cytokines including IL-17 and TNFα. It is suggested that the

mechanism of activation impacts the inflammatory program of NCR+ILC3. While cytokine stimulation largely results in the production of IL-22 and GM-CSF, engagement of NKp44 on the cell surface induces a pro-inflammatory signature and aberrant TNFα and IL-2 (Glatzer et al., 2013).

ILC have not been previously investigated in JIA, although NCR+ILC3 have been reported in the skin, blood and synovial fluid from patients with adult psoriatic arthritis where they were associated with more severe disease (Teunissen et al., 2014b, Leijten et al., 2015). Additionally, an enrichment of NCR+ILC3 have been detected in the blood, synovial fluid and ilium of adult ankylosing spondylitis (AS) patients (Ciccia et al., 2012, Ciccia et al., 2015).

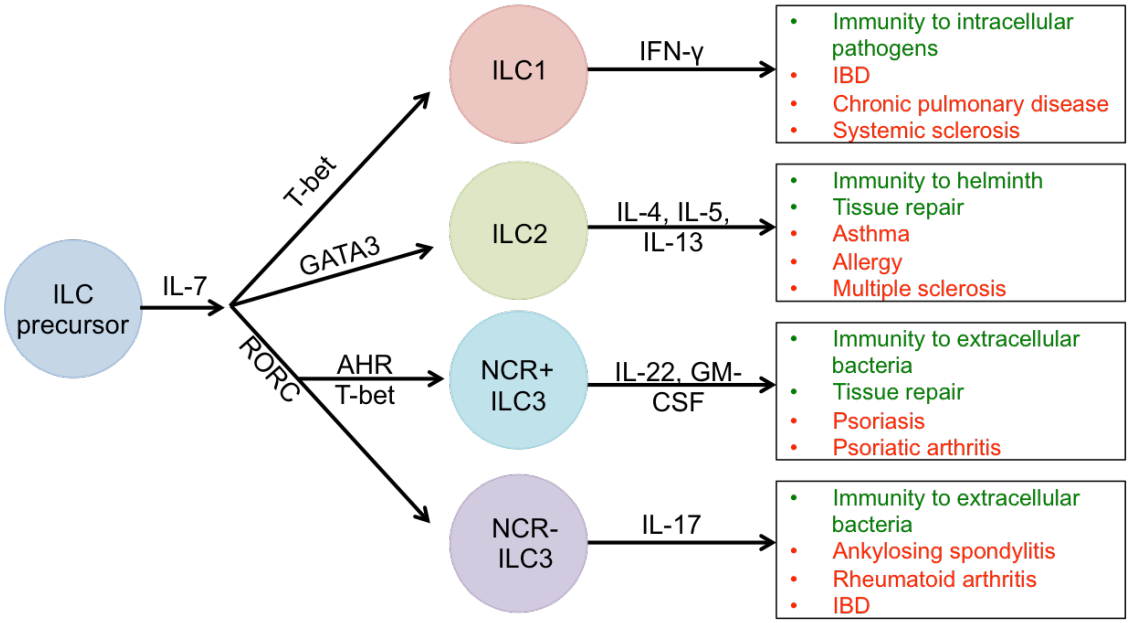


Figure 1.1 Schematic showing ILC subtypes and disease associations.

1.2.1.1.3 ILC plasticity

Adding complexity to this population of cells, there is now significant evidence, in both human and mouse systems, that there is substantial plasticity between ILC subpopulations. This plasticity allows ILC groups to switch phenotype (Bernink et al., 2013b, Klose et al., 2014). Skewing of human ILC1 to ILC3 has been clearly shown under the influence of IL-23 and IL-1β (Bernink et al., 2015). It is additionally clear that this plasticity is reversible and under the influence of

IL-12, ILC3 populations are able to up regulate expression of Tbet and initiate production of IFNγ, essentially transitioning back into ILC1 (Bernink et al., 2013b). One example of this in a disease setting is the depletion of intestinal mucosal NCR+ILC3 in Crohn's disease patients, which is likely to be a result of IL-12 dependent switching to ILC1. Other mediators of ILC switching include: retinoic acid, which causes switching towards IL-22 producing ILC subsets (Bernink et al., 2015). In the presence of IL-23 and IL1β, NCR-ILC3 can transdifferentiate into NCR+ILC3. There is even increasing evidence to support ILC2 plasticity, whereby IL-4 induces ILC2 differentiation and IL-12 and IL-1β induces switching towards an ILC1 phenotype (Silver et al., 2016b). Intriguingly, while stimulation with IL-23 causes IL-17 and IL-22 production from ILC3 populations, extended exposure can promote a more pathogenic phenotype and IFNγ production (Mielke et al., 2013, Bernink et al., 2013b). Together these reports highlight the sensitivity and versatility of CD127+ILC and their ability to readily adapt to their inflammatory environment.

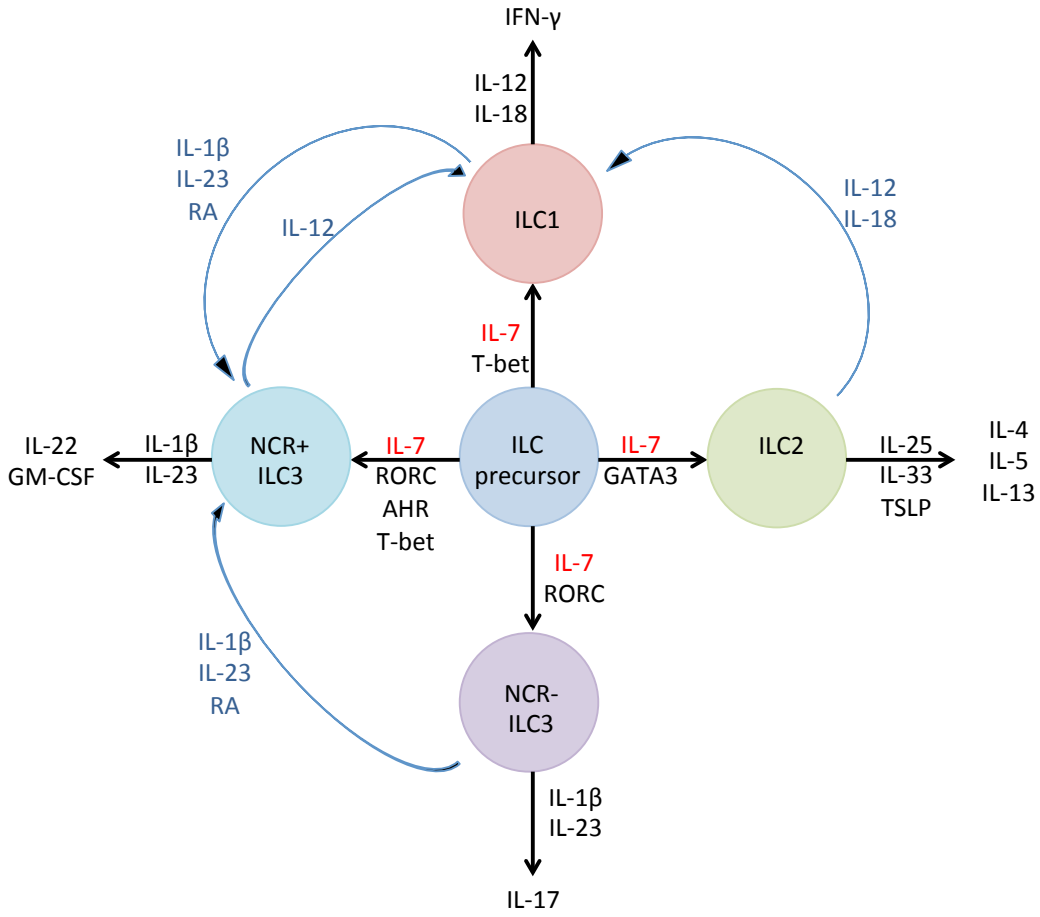


Figure 1.2 Schematic showing ILC differentiation and plasticity.

1.2.1.2 Myeloid dendritic cells

Myeloid dendritic cells (mDC) are a bone marrow derived population, which form an essential arm of the innate immune system and function as APC, characteristically identified by the expression of CD11c and CD13 in the absence of CD14. mDC can be further divided into two groups according to the expression of BDCA1 (CD1c) and BDCA3 (CD141) respectively(Dzionek et al., 2000). The primary function of mDC is antigen uptake and subsequent presentation to T cells in secondary lymphoid tissues.

Immature mDC are typically localized in peripheral tissues and have high endocytic capacity, but a low capacity for T cell activation. As APCs, mDC continuously sample and internalize antigens, employing a combination of micropinocytosis, macropinocytosis and phagocytosis. While micro- and macro-pinocytosis do not require antigen recognition, phagocytosis relies on the identification of foreign antigens by MAMPs such as Toll-like receptors and other microbial sensors on the cell surface (reviewed in (Cella et al., 1997)).

Internalised antigens are degraded within lysosomes and the constituent peptides are subsequently loaded into MHC class II molecules and presented on the cell surface to MHC-class II-restricted CD4 T cells. Additionally, intracellular pathogens, and phagocytosed material that has escaped lysosomes and moved into the cytosol, do not escape processing by mDC. These antigens are processed in a pathway known as “cross presentation”. During cross presentation, antigens are degraded within the proteasome and loaded onto MHC class I molecules. In this way mDC may also interact with and activate CD8 T cells, which are MHC-class I-restricted (Norbury et al., 1995, Reis e Sousa and Germain, 1995).

Upon activation by antigen uptake, or from local cytokines, including TNFα and IL-1, mDC mature and their function transitions from antigen capture, to antigen presentation. At this time, the cells upregulate expression of chemokine receptor, CCR7, as well as several co-stimulatory molecules, such as CD80 and CD86, and migrate towards the T cell zones of local lymph nodes and

secondary lymphoid tissues. The CCR7 receptor allows migration of cells towards these lymphoid tissues (reviewed in (Forster et al., 2008)). Here, they may present processed peptides to circulating naïve T cells via their MHC molecules and high expression of co stimulatory molecules such as CD80 and CD86, required to activate naïve T cells. In parallel, mDC also secrete a plethora of cytokines. The combination of cytokines secreted, combined with antigen presentation, works to drive clonal expansion of T cells and direct the nature of the immune response elicited (reviewed in (Cella et al., 1997)). In addition to their role in antigen presentation, mDC and their cytokines have been implicated in promoting inflammation and autoimmunity. Of particular note, mDC are implicated in the pathogenesis of HLA-B27 mediated disease, via aberrant production of IL-1β and IL-23, supporting pathogenic Th17 responses (Goodall et al., 2010).

Interestingly, tolerogenic mDC have now been described (Coombes et al., 2007, Xia et al., 2008), although these cells have not been investigated in JIA. Rather than exacerbating inflammation, these cells are important for the control of aberrant immune responses and the maintenance of immune homeostasis. Of particular interest in the context of this thesis is a population of mucosal mDC defined by expression of integrin αE (CD103), which is encoded by *ITGAE*(Kilshaw, 1999). CD103+mDC are major producers of retinoic acid (RA) in the gut under homeostatic conditions and are important for the induction of lymphocytes with gut homing properties (Iwata et al., 2004, Johansson-Lindbom et al., 2005, Coombes et al., 2007, Jaensson et al., 2008). Compared to CD103-mDC, CD103+mDC express high levels of retinal dehydrogenase enzymes, which are essential catalysts for the conversion of vitamin A to active RA(Coombes et al., 2007, Hall et al., 2011). RA subsequently acts in an autocrine positive feedback mechanism to further enhance RALDH expression and augment RA catalysis(Molenaar et al., 2011). RA is an important immune mediator and has been shown to impact the development and stability of T cell lineages(Hall et al., 2011). Of note, RA, in combination with TGFβ, is shown to stimulate the differentiation of regulatory T cells(Benson et al., 2007, Mucida et al., 2007, Xiao et al., 2008). Meanwhile, high dose RA inhibits the development of pathogenic Th17 responses(Mucida et al., 2007, Xiao et al., 2008). More

recently, as mentioned above, RA has also been shown to augment ILC skewing and support the development of IL-22-producing NCR+ILC3(Bernink et al., 2015).

1.2.2 Adaptive immunity

Members of the adaptive immune system are defined by expression of highly specific, RAG-recombined antigen recognition receptors. The adaptive response can be divided into T- and B- lymphocytes, both of which are able to develop memory and thus respond quickly and efficiently to repeated antigen exposure. As T cell responses were investigated in detail in this thesis, B cell function is not discussed further.

1.2.2.1 T cells

Two main populations of T cells exist, defined by the chains that constitute the heterodimeric TCRs that they express. $\alpha\beta$ T cells (CD4+ and CD8+) represent the majority of T cells in human blood and express TCRs made up of an alpha-chain and beta-chain, while $\gamma\delta$ T cells represent the minority of T cells within the blood, and express TCR constituted of a gamma-chain and delta-chain(Marrack and Kappler, 1986, Brenner et al., 1986). T cells, similarly to ILC, are derived from common lymphoid progenitor cells; which arise from hematopoietic stem cells in the bone marrow and subsequently migrate to the thymus. Within the thymus, these committed progenitor cells (thymocytes) which lack CD4 and CD8 expression, differentiate into CD4+ and CD8+ and $\gamma\delta$ T cell subsets via several stages of positive and negative selection. In order to generate TCR specificity and diversity, TCR genes undergo RAG-dependent recombination of the variable (V), diversity (D) and joining (J) gene regions (Tonegawa et al., 1981). While V(D)J rearrangement results in enhanced diversity, the most significant contribution to TCR diversity arises from the addition or removal of nucleotides at the junctions between V and D and J segments, which is known as junctional diversity. After successfully undergoing selection, naïve T cells will finally migrate out of the thymus and into the periphery where they may

differentiate further and perform their effector functions, which vary according to T cell subtype.

1.2.2.1.1 CD4 T cell subsets

CD4 T cells, commonly called T helper (Th) cells, comprises a heterogeneous T cell population whose main effector functions aid in guiding the immune response via antigen recognition and cytokine production. Th1 and Th2 were first to be identified as distinct populations, with divergent signature cytokines and effector functions (Mosmann et al., 1986). Since then, many other Th subsets have been reported and can be distinguished according to their cytokine and transcription factor profiles (Harrington et al., 2005, Crotty, 2014, Kaplan et al., 2015). Differentiation of CD4 T cells into their Th subtype is directed by antigen recognition and the local cytokine milieu. This thesis will focus predominantly on Th1, Th17 and regulatory CD4 T cells (Treg) and therefore only these populations will be discussed further in this introduction.

1.2.2.1.1.1 Th1

Th1 cells are important for immunity to intracellular bacteria, and are heavily implicated in autoimmunity. Th1 cells are regulated by the master transcription factor Tbet and readily produce IFNγ, IL-2 and TNFα upon activation (Mosmann et al., 1986, Szabo et al., 2000). Th1 differentiation is driven by IL-12, which signals via the STAT4 pathway(Bacon et al., 1995). IFNγ production is induced by activation by IL-12 in combination with IL-18 or, to a lesser extent, IL-1β (Tominaga et al., 2000). Additionally, IFNγ secreted by Th1 cells operates in a positive feedback mechanism to further enhance production of IFNγ, whereby STAT1 is activated resulting in increased transcription of Tbet, which induces increased expression of IFNγ and expression of the inducible chain of the IL-12 receptor (Lighvani et al., 2001). In parallel, ligation of CD40 ligand on Th1 cells with CD40 on the surface of macrophages and DC results in increased IL-12 secretion by APCs and exacerbates inflammation. In addition to their cytokine

profile, Th1 cells can also be characterized by expression of chemokine receptors CXCR3 and CCR5 on their surface (Loetscher et al., 1998, Bonecchi et al., 1998).

1.2.2.1.1.2 Th17

Th17 cells are most abundant in mucosal sites, where they offer protection against extracellular bacterial and fungal infections. Defined by production of IL-17 (A and F) and regulated by expression of the transcription factor, RORC, Th17 cells were first described in murine models of autoimmunity (Ivanov et al., 2006). In this context, Th17 cells were shown to be critical for the onset of experimental autoimmune encephomyelitis, and have since been implicated in the pathogenesis of a plethora of inflammatory diseases including mouse and human arthritis and diabetes (Langrish et al., 2005, Spreafico et al., 2016).

In mice, Th17 differentiation is driven by various combinations of; IL-23 (which shares a common subunit, (p40) with IL-12) with: IL-6, transforming growth factor β (TGFβ) and IL-1β. There are conflicting reports on the factors, which drive Th17 differentiation in humans. While some studies have suggested a cocktail of IL-6 and IL-1β is sufficient; other studies suggest that addition of TGFβ and IL-23 is also required. In addition to the cytokine milieu, weak TCR signalling has been shown to promote Th17 differentiation (Mangan et al., 2006, Veldhoen et al., 2006, Acosta-Rodriguez et al., 2007a, Wilson et al., 2007, Volpe et al., 2008).

There are a number of surface molecules that are associated with the Th17 lineage. Natural killer lectin-like receptor CD161 is highly expressed on Th17 cells and is induced by RORC expression. In fact, CD161 is now considered to be a marker of all IL-17-producing T cells and is identified on IL-17-producing T cell subsets as well as naïve T cell precursors with the potential for IL-17 production(Maggi et al., 2010). Additionally, Th17 cells commonly express chemokine receptors CCR4 and CCR6(Acosta-Rodriguez et al., 2007b).

1.2.2.1.1.3 Regulatory T cells

In addition to facilitating inflammation, Th cells, known as Treg, also play an important role in suppressing aberrant immune responses and controlling the inflammatory process. Treg produce anti-inflammatory cytokines such as IL-10. Other mechanisms of Treg suppression include inducing apoptosis of activated lymphocytes and inhibition of T cell activation via CTLA4-mediated interactions (Zheng et al., 2008, Walker and Sansom, 2011, Qureshi et al., 2011). CD25+ (IL-2 receptor alpha chain) CD4+ regulatory T cells (Treg) were first described by Sakaguchi, who noted that autoimmunity was prevented in athymic mice inoculated with effector T cells and subsequently reconstituted with CD4+CD25+ T cells(Sakaguchi et al., 1995). Since then, Treg have been extensively investigated in mice and in humans, where they can be identified by low expression of CD127 high CD25 expression and the transcription factor forkhead box P3 (FOXP3)(Baecher-Allan et al., 2001, Fontenot et al., 2003). Additionally, committed Treg can be distinguished from effector T cells transiently expressing FOXP3 by demethylation of the Treg-specific demethylated region (TSDR)(Baron et al., 2007).

To add to the complexity, there is now also evidence of Treg populations with effector functions. Populations of Treg have been identified by divergent expressions of T-cell immunoreceptor with Ig and ITIM domains (TIGIT) and CD226, with Th1- and Th17-like properties, including the expression of CXCR3, T-bet, CCR6 and RORC and production of inflammatory cytokines including IFNγ(Joller et al., 2014, Fuhrman et al., 2015). Other effector Tregs can be identified by expression of CD161(Pesenacker et al., 2013). CD161+Treg represent a true Treg population. They have been shown to be predominantly demethylated at the TSDR and to be suppressive in vitro. However, unlike traditional (CD161-)Treg populations, CD161+Treg, are able to produce a plethora of pro-inflammatory cytokines. Furthermore, CD161+Treg share a transcriptional signature with CD161+ Th1 and Th17 cells(Duurland et al., 2017a). Intriguingly, the CD161+ Treg population is expanded at the inflamed site in oligo-JIA patients, although it is currently unknown whether they are playing a pro- or anti-inflammatory role in this setting. However cytokine

producing Treg have been identified in autoimmune hepatitis where they are implicated in promoting inflammation (Arterbery et al., 2016).

1.2.2.1.2 T cell plasticity

Not long after the identification of Th subsets, it became clear that these defined lineages were not clear-cut. It is now evident that there is considerable plasticity within the Th17 subset, as IL-17 production by Th cells is often paired with production of other inflammatory mediators including IFNγ, IL-22 and GM-CSF. Th cell plasticity is driven by a combination of cytokine stimulation, the microenvironment and epigenetic modifications (Bonelli et al., 2014, Geginat et al., 2014).

Th17 plasticity is widely reported at sites of inflammation. Previous members of the Wedderburn group have described Th cells in the inflamed joints of JIA patients, with a phenotype between Th1 and Th17(Nistala et al., 2010a). These Th1/Th17 cells readily produce both IFNγ and IL-17A and express gene transcripts for Tbet and RORC. This study also showed that culture of Th17 with IL-12 induced phenotype switching towards Th1 in vitro. A subsequent study confirmed this finding, and additionally showed that IL-12 stimulation also induced production of GM-CSF from Th17(Piper et al., 2014a). Whereby Th1 and Th17 cells characteristically express CXCR3 and CCR6 respectively, Th1/Th17 intermediate cells can be identified by the co-expression of both of these chemokine receptors (Acosta-Rodriguez et al., 2007b). In addition to IL12, IL-1β is shown to be important for the differentiation of these pathogenic Th cells. In addition to demonstrating Th17 plasticity, Nistala *et al* also identified CD161 as a maker of Th1 cells with the ability to produce IL-17, so-called “ex Th17” cells (Nistala et al., 2010a).

Th17 plasticity is not only associated with a Th1 phenotype. Cells with a Th17/Th2 intermediate phenotype have been described in the peripheral blood of asthma patients, and this phenotype can be induced by IL-4 stimulation (Cosmi et al., 2010). Finally, plasticity between Th17 and Treg may also exist,

and differentiation of Th17 cells to Tregs and vice versa has been demonstrated in mouse models (Komatsu et al., 2014, Gagliani et al., 2015). Although this plasticity has not been confirmed in human studies, CD161 expression is associated with a Treg population with effector, Th17-like, function in humans and supports parallel plasticity (Pesenacker et al., 2013, Duurland et al., 2017a).

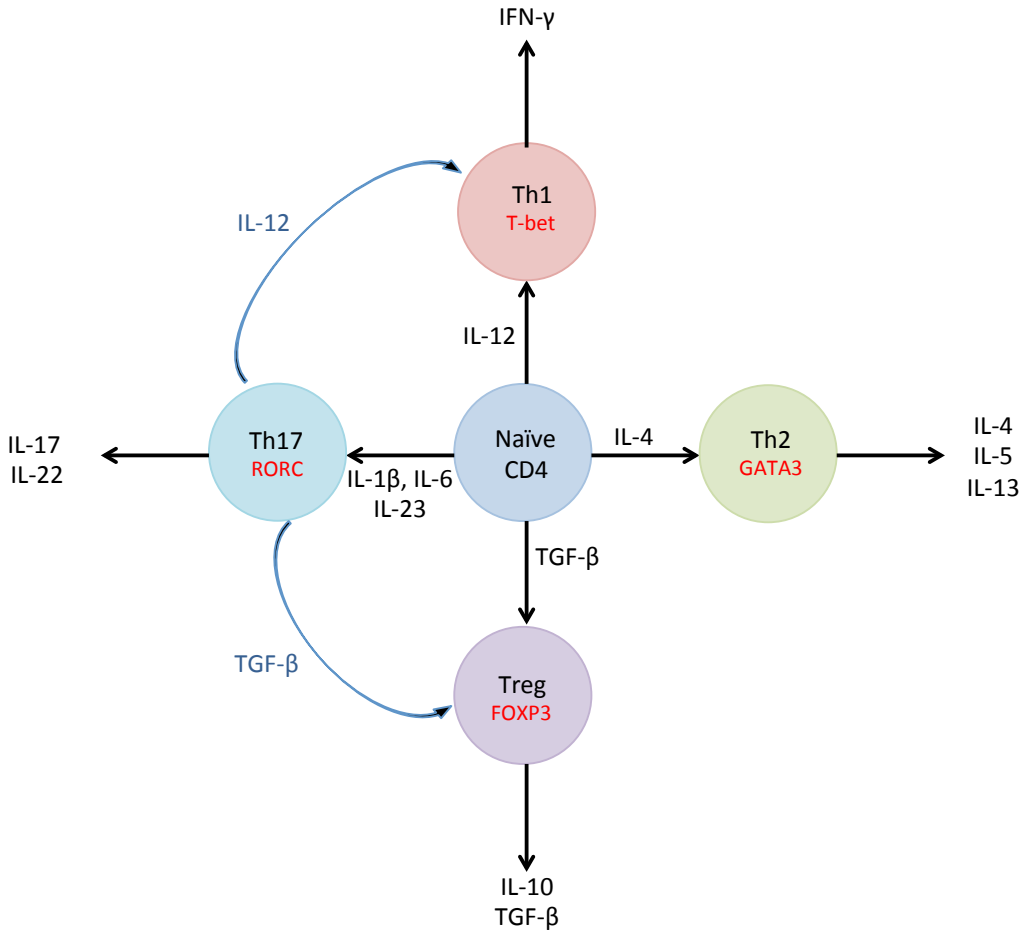


Figure 1.3 Schematic showing T cell differentiation and plasticity.

1.2.2.2 CD8 T cells

Defined by expression of CD8 on their surface, cytotoxic T lymphocytes (CTL) are key mediators of immunity against intracellular pathogens. CD8 T cells recognize antigens presented on MHC class I molecules and their subsequent activation requires costimulation. Although CD8 T cells can respond without the help of CD4 T cells, additional CD4-derived cytokines and CD4 T cell licensing of APC may boost the CD8 T cells responses(Bennett et al., 1997).

The primary function of CD8 T cells is to kill infected or damaged cells. Upon activation, CTL acquire the machinery for efficient killing of target cells such as cytoplasmic granules containing cytotoxic proteins including perforin and granzymes, which, when injected into cells expressing the appropriate antigens, induce apoptosis(Chowdhury and Lieberman, 2008). CD8 T cells are also able to mediate killing in a granule-independent mechanism. Upon activation CD8 T cells upregulate of FAS ligand (FASL) on the cell surface. Ligation of this receptor results in caspase activation and apoptosis of the cognate cells(Rouvier et al., 1993a).

In addition to killing functions, CD8 T cells also contribute to the inflammatory process via the production of cytokines. Most commonly CD8 T cells produce IFNγ, which functions to activate phagocytes and prolong the inflammatory loop, however subsets producing IL-17A and other cytokines have now been described. Similarly to Th cells, CD8 T cell subsets can be categorized according their transcription factor, cytokine and chemokine receptor profiles, which mirror their CD4 counterparts described above (Croft et al., 1994, Mosmann et al., 1997, Hamada et al., 2009).

Of relevance to this thesis, a new population of innate-like CD8 T cells expressing high levels of CD161, a marker associated with IL-17A production have been identified, known as mucosal associated invariant T (MAIT) cells (Treiner et al., 2003). These cells are potent IL-17A-producers and have been identified in a plethora of inflammatory conditions (Cho et al., 2014, Willing et al., 2014, Serriari et al., 2014b). Additionally, MAIT cells have been implicated in adult arthropathies where they contribute to IL-17-mediated disease (Gracey et al., 2016).

1.2.2.3 γδ T Cells

T cells expressing the γδ TCR heterodimer constitute less than 5% of the T cell repertoire in peripheral blood, although γδ T cells are more abundant in epithelial surfaces, specifically the small intestine(Viney et al., 1990). Unlike

their αβ counterparts, antigen recognition by γδ T cells is typically not MHC-restricted. Instead these cells function in a more innate-like way, recognizing conserved antigens using PRRs and antigens which are presented on MHC-like molecule, CD1c, via their TCRs. γδ T cells typically home to tissues rather than lymphoid organs where they respond in harmony with the innate immune system.

γδ T cells have diverse functions (reviewed in (Vantourout and Hayday, 2013)). These cells may kill infected or damaged cells via interaction of FAS and the release of cytotoxic agents. Additionally, γδ T cells are potent cytokine producers. In response to activation, γδ T cells may secrete inflammatory, or modulatory cytokines including IFNγ, IL-6, IL-17A, GM-CSF and TNFα, all of which have been implicated in autoimmune pathogenesis. Although TCR stimulation is not required for all function, there is now some evidence that IL-17A production from γδ T cells requires TCR ligation(MacLeod et al., 2013). There is evidence, as with the aforementioned T cell subpopulations, of γδ T cell functional plasticity although this has not been studied in great detail in humans.

Although γδ T cell function is relatively poorly understood in humans, their contribution to maintaining immune competence should not be over looked. It has been shown that γδ T cells are rapidly reconstituted after transplantation, where their population size correlated with disease-free survival(Wu et al., 2000, Godder et al., 2007). Additionally, there is now growing evidence to support a role for pathogenic γδ T cells in promoting autoimmune disease(Bank et al., 2002).

1.2.3 Chemokine mediated cell trafficking

Chemokine proteins are important mediators of leukocyte trafficking, which instruct cellular migration via a concentration gradient. Leukocytes expressing the cognate chemokine receptors move along the chemokine concentration gradient towards the epicentre, where they may perform their functions(Charo

and Ransohoff, 2006). The two major families of chemokines are identified according to the localization of cysteine residues within their structure. Within CC chemokines, the first two cysteine residues are adjacent, whereas CXC chemokines have a single amino acid residue between their first two cysteine residues(Zlotnik and Yoshie, 2000). Chemokines from both of these families signal through chemokine receptors containing a seven-transmembrane-domain coupled to a G-protein(Holmes et al., 1991). Ligation of chemokines with their associated receptors results in conformational and morphological changes within the cell that enables movement.

Chemokines may perform homeostatic or inflammatory functions(Charo and Ransohoff, 2006). CCR7, which migrates towards CCL19 and CCL21, is commonly expressed on naïve T cells and is important for their migration through lymphoid tissues and the maintenance of homeostasis(Campbell and Butcher, 2000). However, high levels of other specific chemokines have been reported in inflamed sites and in autoimmune diseases, paired with increased expression of their receptors on immune cells. In this position, chemokines maintain and exacerbate inflammation. Table 1.2 shows selected chemokine receptors, their typical expression and their respective ligands.

Chemokine receptor	Chemokine ligands	Cell type expressing receptor
CCR4	CCL2, CCL4, RANTES, CCL17, CCL22	Th2, Th17, Treg, mDC, basophils, macrophages
CCR5	CCL3, CCL4, RANTES, CCL11, CCL14, CCL16	Th1, monocytes
CCR6	CCL20	Th17, gut homing T cells, immature mDC
CCR7	CCL19, CCL21	Naïve T cells
CXCR3	CXCL4, CXCL9, CXCL10, CXCL11	Th1, NK cells

Table 1.2 Selected chemokine receptors, their ligands and typical expression.

1.2.4 The functions of IL-17 and associated cytokines

1.2.4.1 IL-17A

IL-17 denotes a family of cytokines including, IL-17A-F. Of these, the most thoroughly understood, and in focus in this thesis, is IL-17A, which has been implicated in mediating the pathogenesis of numerous inflammatory diseases and is an increasingly prominent target of therapy (unless otherwise specified, IL-17 will refer to IL-17A throughout this thesis)(Agarwal et al., 2008b, Henriques et al., 2010, Omoyinmi et al., 2012, Jansen et al., 2015, Gaur et al., 2015). IL-17A was initially discovered in 1993, and comprises a homodimeric, disulphide-linked, glycoprotein of 155 amino acids(Rouvier et al., 1993b). Most often associated with Th cells, IL-17A is also produced by other cells of the innate and adaptive immune systems including γδ T cells, CD8 T cells and ILC(Ivanov et al., 2006, Michel et al., 2007, Roark et al., 2008, Hamada et al., 2009). There are currently 5 known members of the IL-17 receptor family (IL-17RA-E), and signalling of IL-17A requires a heterodimer receptor complex comprising IL-17RA and IL-17RC. Binding of IL-17A to the cognate receptors activates NFκB and MAPK in a TRAF-dependent cascade and triggers a highly pro-inflammatory gene expression program (reviewed in detail in (Gaffen, 2009)).

IL-17A is a potent inflammatory mediator, which acts predominantly on epithelial cells, B cells, T cells, synovial fibroblasts and vascular endothelial cells (Hwang et al., 2004), (Ouyang et al., 2008) IL-17A is known to be important in protection against fungal and bacterial infections e.g. C*andida albiocans* (Conti et al., 2009) and *Klebsiella pneumoniae* (Happel et al., 2003). IL-17A functions to strengthen epithelial tight junctions in the gut (Kinugasa et al., 2000), which suggest that IL-17A is important for immune defence at mucosal surfaces. In the context of arthritis, IL-17A has been shown to be important for the production of RANKL, which contributes to the formation of bony erosions, as well as the induction of pro-inflammatory cytokine secretion from monocytes and synovial fibroblasts (Chabaud et al., 2001).

1.2.4.2 IL-22

The functions of IL-22 are diverse and appear to be context dependent (Wolk et al., 2004). IL-22 plays important roles in promoting defence mechanisms by strengthening epithelial barrier functions, in wound healing and in supporting tissue homeostasis (Boniface et al., 2005, Nikoopour et al., 2014). On the other hand, IL-22 can also play an inflammatory role; chronic IL-22 exposure can lead to pathology. In this context, IL-22 has been shown induce epithelial hypoplasia in psoriasis, and increased serum levels have been associated with poorer prognosis in rheumatoid arthritis where it acts to induce release of inflammatory cytokines by synovial fibroblasts (Ikeuchi et al., 2005, Leipe et al., 2011).

IL-22 belongs to the IL-10 family of cytokines and signals through a receptor composed of two subunits; the IL-10R2 and IL-22R1(Xie et al., 2000). IL-22 signalling downstream of the receptor is STAT3 dependent. IL-22 acts predominantly on non-immune cells such as epithelial cells and hepatocytes rather than leukocytes(Nagalakshmi et al., 2004).

Members of both the innate and adaptive immune systems express IL-22, although it is most commonly associated with T cells and more specifically; Th17 cells (Liang et al., 2006). However, while the association of IL-17 and IL-22 production by T cells is strong in the mouse, the association is much weaker in humans, where only 10-15% IL-22 producing CD4 T cells co-express IL-17, supporting the existence of a separate Th22 subset in humans (Eyerich et al., 2009). In humans, IL-22-producing CD4 T cells are primed by IL-6 and TNFα and express only low levels of RORC, however additional culture with IL-1β is shown to induce IL-17A production demonstrating significant plasticity between these two subsets (Bachmann et al., 2010). Another significant source of IL-22 are ILC. Unlike T cells, ILC expressing RORC commonly co-express IL-17 and IL-22, although IL-22 single producing ILC also exist (Hazenberg and Spits, 2014a).

1.2.4.3 Granulocyte-macrophage colony stimulating factor (GM-CSF)

GM-CSF is a pro-inflammatory cytokine now associated with pathogenic Th17 cells (El-Behi et al., 2011). GM-CSF is produced by a number of cell types in addition to T cells including; monocytes, fibroblasts and endothelial cells (Burgess et al., 1977, Blanchard et al., 1991). GM-CSF signals through a heterodimeric receptor (GM-CSFR) composed of a specific α-subunit and signal-transducing β-subunit. Downstream of the receptor, signalling activates JAK2 and STAT5. The GMSCF receptor is predominantly expressed on DC and monocytes as well as other myeloid cells(Matsuguchi et al., 1998).

The primary role of GM-CSF is as a growth factor, pivotal for the differentiation of myeloid cells, including CD103+mDC (King et al., 2010). More recently, GM-CSF has also been implicated in mediating inflammation. GM-CSF is thought to influence the inflammatory activity of neutrophils by inducing formation of neutrophil extracellular traps (NETs) and is also shown to promote the differentiation of inflammatory macrophages (Akagawa et al., 2006, Yousefi et al., 2009, Rasouli et al., 2015). In murine models of inflammatory arthritis and multiple sclerosis (MS), GM-CSF has been shown to be crucial for disease development as GM-CSF-knock out animals were resistant to disease induction(Codarri et al., 2011). The precise role for GM-CSF in human inflammatory disease has not been well explored. However, recent studies have identified expansions of GM-CSF-positive T cells at the inflamed site in rheumatoid arthritis, oligo-JIA and MS, where expression overlapped significantly with IL-17A production (Piper et al., 2014b). GM-CSF blockade is now also in trials for the treatment of rheumatoid arthritis and early reports have shown promise (Burmester et al., 2017).

1.3 Aims of this thesis

While a Th17 phenotype has been described in other subtypes of JIA, little is known about the immune pathology of ERA JIA. This thesis investigates the role of IL-17-producing immune cells in disease pathogenesis, comparing the ERA subtypes to other subtypes of JIA, and with a specific focus on T cells and innate lymphoid cells.

To begin these analyses, this thesis will:

1. Examine cytokine production by T cells within the PBMC and SFMC from JIA patients and healthy controls.
2. Explore the ILC signature at the inflamed site in JIA patients.
3. Investigate the mechanisms by which ILC are selectively recruited to and maintained within the joints of JIA patients.

2 : Materials and Methods

2.1 Sample preparation

2.1.1 Sample collection

Children with all clinical subtypes of JIA, diagnosed according to International League of Associations for Rheumatology; ILAR; (Petty et al., 2004), were recruited to an on-going study investigating the pathogenesis of JIA (95RU04). Informed parental consent and age-appropriate assent were obtained. Patient and control demographics and clinical details are listed in Table 2.1.

Synovial fluid (SF) was aspirated from the joints of JIA patients at the time of therapeutic intra-articular steroid injections. A small volume of extra peripheral blood was collected for research purposes when routine clinical blood tests were carried out. Blood samples were also obtained from healthy children and teenagers at local schools as part of a public involvement program instigated by the Arthritis Research Centre for Adolescent Rheumatology. In addition, peripheral blood samples from adult healthy volunteers at UCL GOS ICH with full informed consent were also obtained. All samples were anonymised by the allocation of codes with specific prefixes according to healthy status or JIA subtype, or CW for healthy child, AW for healthy adult. Informed consent was obtained from each patient and healthy control in accordance with the local ethics approval committee of Great Ormond Street Hospital and the Institute of Child Health (ref. 95RU04 and 07RU01).

	Adult healthy control (n=20)	Child healthy control (n=4)	Oligo-arthritis (n=47)	Polyarticular arthritis (n=7)	Enthesitis related arthritis (n=42)	Psoriatic arthritis (n=5)
No. male/female	8/12	4/0	10/37	3/4	41/1	2/3
Age at sampling (years), median (IQR)	26.15 (23.97-30.36)	6.24 (5.48 – 8.31)	8.45 (5.70 – 10.66)	11.22 (8.74 – 13.66)	13.22 (11.71 – 15.42)	13.52 (12.11 – 14.29)
Age at disease onset (years), median (IQR)	n/a	n/a	3.27 (1.68 – 5.68)	2.42 (1.89 – 6.09)	10.00 (7.87 – 12.38)	10.97 (8.93 – 10.97)
Treatment received within preceding 6 months of sample: MTX (%)	n/a	n/a	16/47 (34.04%)	6/7 (85.71%)	25/42 (59.51%)	3/5 (60%)
Treatment received within preceding 6 months of sample: Biological therapy (%)	n/a	n/a	0/47 (0%)	1/7 (14.29%)	1/42 (2.38%)	0/5 (0%)
No. of swollen/tender joints involved at time of sampling, median (IQR)	n/a	n/a	1 (1 -2)	3.5 (1.5 – 4.75)	2 (1 -3)	2 (2 – 3.5)
ESR mm/hr at time of sampling, median (IQR)	n/a	n/a	27 (12.5 – 52.5)	86 (72 – 94)	32 (15.5 - 50)	16 (14 – 25.5)
HLA-B27+ (%)	n/a	n/a	n/a	n/a	33/42 (78.57 %)	n/a
RF + (%)	n/a	n/a	n/a	2/7 (28.6%)	n/a	n/a

Table 2.1 Clinical details of JIA patients recruited to this study

2.1.2 Isolation of Peripheral Blood Mononuclear Cells (PBMC)

Peripheral blood was collected into sterile universals containing 35µl preservative-free sodium heparin (PFH) (Sigma). Samples were processed in a sterile fume hood within 4 hours of collection using sterile, endotoxin- free reagents and tissue culture grade plastics. An equal volume of RPMI 1640

medium supplemented with Penicillin (100units/ml) and Streptomycin (100µg/ml) (RPMI) (all Gibco) was added to the blood sample. In the ratio of 2:1, the diluted blood was gently layered onto lymphoprep (Axis-Shield) for density centrifugation. To ensure minimal mixing of the layers, samples were centrifuged (800g for 20 minutes) with no brake. The PBMC-containing layer at the interface of the blood and lymphoprep was carefully aspirated, added to an equal volume of RPMI containing 10% foetal calf serum (FCS) in RPMI (10%RPMI), and centrifuged (500g for 10 minutes). The supernatant was discarded and the cell pellet was resuspended in 10% RPMI. A 10µl aliquot was removed for cell counting. Finally the cells were centrifuged (300g for 7 minutes) and frozen at -80°C and then stored in liquid nitrogen (see below) until required.

2.1.3 Isolation of Synovial Fluid Mononuclear Cells (SFMC)

Synovial fluid was diluted in an equal volume of pre-warmed 10% RPMI, and hyaluronidase (Sigma) was added to a final concentration of 10U/ml. This mixture was incubated at 37°C for 30 minutes to reduce the viscosity of synovial fluid and prevent cells clumping. The SF mix was then carefully layered onto lymphoprep and SFMC were isolated following the same protocol as described for PBMC isolation.

2.1.4 Counting viable cells

To quantify viable cells, an aliquot of the cell suspension was diluted in an equal volume of 0.4% Trypan blue (Sigma). After mixing, 10µl was transferred to a Neubauer counting chamber and viable cells were counted. Dead cells (stain blue due to dye influx) were also counted for sample viability calculation. Samples with less than 90% viable cells were discarded. To enumerate live cells in a sample the following formula was employed:

Number of cells counted in 25 square box x 2 (dilution factor) x total volume of cell suspension (ml) x 10^4 (factor for counting chamber) = Total viable cell number.

2.1.5 Freezing and thawing of cells

To freeze isolated PBMC/SFMC: the cell suspension was centrifuged (300g for 7 mins) as described in 2.1.2. The cell pellet was carefully resuspended in filtered freezing medium (10% DMSO, 90% FCS) at a final concentration of $10x10^6$ cells per ml freezing medium. The cells were then aliquoted into cryovials (NUNC) and slowly frozen to -80°C in a freezing pot containing isopropanol (Nalgene), allowing a typical temperature drop of 1°C per hour. The next day samples were transferred to liquid nitrogen.

To thaw: cryo-preserved PBMC/SFMC were retrieved from liquid nitrogen and rapidly thawed in a water bath at 37°C. The thawed cells were added to pre-warmed 10%RMPI. An aliquot was taken for cell enumeration, and the remaining mixture was centrifuged (300g for 7 minutes). Finally, the cell pellet was carefully resuspended in an appropriate volume of the appropriate medium for subsequent use.

2.1.6 Serum and plasma preparation

For serum samples and equivalent synovial fluid samples, 1ml venous blood or synovial fluid was collected into a sterile plastic tube without anticoagulant. The sample was left for 30 minutes to clot and subsequently spun for 10 minutes at 10,000 rpm in the microfuge. The supernatant was collected, aliquoted, and frozen at -80°C for subsequent use.

For plasma samples, and equivalent synovial fluid samples, 1ml venous blood or synovial fluid was collected into a sterile plastic tube containing EDTA as anticoagulant. The blood/synovial fluid was transferred to an Eppendorf tube and spun in the microfuge for 10 minutes at 1,000rpm. The supernatant was transferred to a clean Eppendorf tube and spun for a further 10 minutes at 10,000 RPM. The supernatant was collected, aliquoted and frozen at -80°C for subsequent use.

2.2 Multiplex cytokine analysis by Luminex

Multiplex cytokine analysis by Luminex analysis of JIA serum samples and JIA SF samples and pools was conducted in Dr de Jager's group at the University of Utrecht.

2.3 Flow Cytometry

2.3.1 Antibodies used

Monoclonal antibodies directly conjugated to fluorescent dyes and polyclonal biotinylated antibodies used in this study are listed in Table 2.2. All new antibodies were titrated to identify the optimal concentration for use. All antibodies used were raised in mouse against human epitopes.

Antibody specificity/ reagent	Fluorochrome	Supplier	Clone	Dilution
Aldefluor	FITC channel	Stem Cell Technologies	n/a	5ul/ reaction
BDCA1	BV421	BioLegend	L161	1 in 50
BDCA2	FITC	MILTENYI	n/a	1 in 50
BDCA2	PE	BioLegend	201A	1 in 50
BDCA3	BV510	BioLegend	1A4	1 in 50
C-KIT	BV421	BioLegend	104D2	1 in 25
CCR4	BV510	BioLegend	L291H4	1 in 25
CCR5	PerCP-Cy5.5	BioLegend	n/a	1 in 25
CCR6	BV510	BioLegend	n/a	1 in 25
CD103	APC	BioLegend	n/a	1 in 50
CD11C	BV421	BioLegend	3.9	1 in 50
CD11C	PE	BioLegend	3.9	1 in 50
CD11C	PE-CY7	BioLegend	n/a	1 in 50
CD123	PE	BioLegend	6H6	1 in 50
CD123	BV605	BioLegend	6H6	1 in 25
CD127	FITC	eBiosciences	EBIORDR5	1 in 25
CD127	BV711	BioLegend	A019D5	1 in 25
CD13	FITC	BD Biosciences	WM15	1 in 50
CD14	FITC	eBiosciences	61D3	1 in 200
CD14	PE	BioLegend	HCD14	1 in 50

CD16	PE	eBiosciences	B73.1	1 in 100
CD161	APC	eBiosciences	HP-3G10	1 in 25
CD161	BV605	BioLegend	HP-3G10	1 in 25
CD161	PECY7	eBiosciences	HP-3G10	1 in 25
CD161	BV421	BioLegend	n/a	1 in 25
CD19	PE	BioLegend	HIB19	1 in 25
CD19	PE-CY7	eBiosciences	SJ25C1	1 in 50
CD1A	PE	eBiosciences	H149	1 in 50
CD25	PE	BD Biosciences	MA251	1 in 25
CD25	BV421	BioLegend	MA251	1 in 25
CD3	PE	BioLegend	UCHT1	1 in 50
CD3	PerCP-Cy5.5	eBiosciences	OKT3	1 in 50
CD3	V500	BD Biosciences	UCHT1	1 in 25
CD3	BV605	BioLegend	OKT3	1 in 50
CD3	BV711	BioLegend	n/a	1 in 50
CD34	PE	BioLegend	561	1 in 50
CD4	BV711	BioLegend	OKT4	1 in 50
CD45	BV421	BioLegend	HI30	1 in 200
CD45	PE-CY7	BioLegend	HI30	1 in 100
CD45RA	FITC	eBiosciences	HI100	1 in 50
CD45RO	PE-CY7	BioLegend	UCHL1	1 in 25
CD45RO	APC	eBiosciences	UCHL1	1 in 50
CD49D	PerCP-Cy5.5	BioLegend	9F10	1 in 25
CD56	PERCP-CY5.5	BioLegend	HCD56	1 in 50
CD56	PE	eBiosciences	CMSSB	1 in 25
CD8a	FITC	BD Biosciences	SK1	1 in 50
CD8a	APC	eBiosciences	SK1	1 in 200
CD94	PE	BioLegend	DX22	1 in 50
CRTH2	FITC	BioLegend	BM16	1 in 25
CXCR3	PerCP-Cy5.5	BioLegend	G025H7	1 in 25
DAPI	DAPI	SIGMA	n/a	1 in 500
FCeR1	PE	BioLegend	AER-37	1 in 100
FOXP3	APC	eBiosciences	236A/E7	1 in 25
GMCSF	PE	BD Biosciences	n/a	1 in 25
HLA-DR	BV510	BioLegend	L243	1 in 50
IFNγ	V500	BD Biosciences	B27	1 in 50
IL-17A	V450	BD Biosciences	N49-653	1 in 25
IL-2	PE-CY7	BioLegend	MQ1-17H12	1 in 100
IL-22	PE	BioLegend	BG/IL22	1 in 10
IL-22	PE-CY7	eBiosciences	22URTI	1 in 25
IL-23R	APC	R&D Systems	n/a	1 in 25
IL-23R	Biotin	R&D Systems	Polyclonal	1 in 10
IL13	APC	BioLegend	JES10-582	1 in 100
IL17A	BV605	BioLegend	BL168	1 in 25
KI67	FITC	eBiosciences	20RAJ1	1 in 25
KI67	PerCP-Cy5.5	eBiosciences	20RAJ1	1 in 25

LIVEDEAD BLUE	DAPI channel	Life Technologies	n/a	1 in 250
NKP44	APC	BioLegend	P44-8	1 in 25
NKP46	PE-CY7	BioLegend	9 e2	1 in 25
PERFORIN	FITC	BioLegend	DG9	1 in 50
RORC	APC	eBiosciences	AFKJS-9	1 in 10
Streptavidin	BV421	BioLegend	n/a	1 in 100
Streptavidin	FITC	BD Biosciences	n/a	1 in 50
TBET	BV711	BioLegend	4B10	1 in 25
TCRαβ	PE	BD Biosciences	n/a	1 in 25
TCRγδ	PE	BD Biosciences	n/a	1 in 25

Table 2.2 Antibodies and staining reagents employed in this thesis.

2.3.2 Surface staining for analysis by flow cytometry

Ex vivo or cultured cells were counted and centrifuged (300g for 7 minutes), and cell pellet resuspended in 10%RPMI at a concentration of 1-5 x 10^6 cells/ml. 100µl of the cell suspension was then plated into wells of a round-bottomed 96 well plate (Thermofisher). For cell concentrations <1x10^6/ml, cells were plated into a V-bottomed plate (Thermofisher). The 96-well plates were then centrifuged (300g for 3 minutes) and the supernatant was removed by flicking. Monoclonal antibodies to the surface epitopes of interest were diluted in FACS buffer (1 x phosphate-buffered saline (PBS) (Gibco) containing 1%FCS and 0.1%sodium azide) and 25µl of antibody mix was added to each of the relevant wells. The plate was then incubated in the dark for 30 minutes at 4°C. 200µl FACS buffer was then added to each well and the plate was centrifuged to wash the cells. Finally the cell pellets were resuspended in 200µl FACS buffer and transferred to FACS tubes (Greiner Bio-One Ltd) and DAPI (Sigma) was added just before cells were acquired on the LSRII flow cytometer (BD Biosciences).

2.3.3 Intracellular and cytokine staining

As described, washed cells were resuspended in 10% RPMI and seeded onto a 96 well plate. To investigate cytokine stimulation, cells were incubated at 37°C

and 5% CO_2 for 4 hours with phorbol myristate acetate (PMA) (50ng/ml) (Sigma-Aldrich), Ionomycin (500ng/ml) (Sigma-Aldrich) and Brefeldin A (5µg/ml) (Sigma-Aldrich). The cells were then washed in 1 x PBS. The cell pellets were subsequently resuspended in Live/Dead fixable blue dead cell stain (Life Technologies), which was first diluted 1 in 250 in 1 x PBS, and incubated in the dark for 30 minutes at 4°C. Following incubation, the cells were washed in FACS buffer and surface markers were stained as described. After staining, the cells were washed with FACS buffer and the cell pellet was resuspended in 100µl Fixation and Permeabilization (Fix/Perm) buffer (1 part fix/perm concentrate, 3 parts fix/perm diluent) (eBiosciences) and incubated in the dark for 45 minutes at 4°C. The cells were then washed in 1 x permeabilization (perm) buffer (eBiosciences). Monoclonal antibodies to the intracellular epitopes of interest were diluted in perm buffer and 25µl of antibody mix was added to each of the relevant wells. The plate was incubated in the dark for 45 minutes at 4°C. The cells were washed in FACS buffer and the cell pellet was resuspended in FACS buffer ready to be acquired on the LSRII flow cytometer.

2.3.4 Analysis of flow cytometry data collected on the LSRII

Data were collected in the LSRII flow cytometer (BD). Maximal events were collected from each sample. Compensation and subsequent analysis was carried out using FlowJo version 10.1 (Treestar Inc.).

2.3.5 Multispectral imaging flow cytometric analysis (ImageStream)

Cells were stained as described above. After fixation, cells were resuspended at $1x10^7$ cells/ml in FACS buffer. Cells were acquired on the Imagestream^X Mark II (Merck Millipore) fitted with 405nm, 488nm and 642nm lasers. Analysis was carried out on the Imagestream^X Mark II instrument.

2.4 Cell sorting

2.4.1 CD3 depletion by magnetic bead cell sorting

For ILC isolation, total PBMC and SFMC were first depleted of T cells using EasySep CD3 positive selection kit (StemCell Technologies) according to the manufacturer's instructions. Samples were thawed, counted and resuspended in sort buffer (1xPBS supplemented with 2% FCS and 4mM EDTA) at a concentration of 1 x 10^8 cells/ml. The cell suspension was transferred to a 5ml polystyrene FACS tube (BD Biosciences), and positive selection CD3 antibody cocktail (100µl/ml cells) was added. Following 15 minute incubation at room temperature (RT), magnetic nanoparticles were added (50µl/ml cells) and mixture was incubated for a further 10 minutes at RT. The mixture was then topped up to 2.5ml with sort buffer and placed into the EasySep magnet for 5 minutes. The supernatant (CD3 negative fraction) was carefully poured into a 15ml falcon tube while the tube remained in the magnet. To ensure maximum yield the tube was washed twice. For this, the tube was topped up with 2.5ml sort buffer and placed in the magnet for a further 5 minutes before the supernatant was removed. Finally the cells were counted as described above.

2.4.2 Dendritic cell negative selection by magnetic bead cell sorting

Myeloid dendritic cells (mDC) were isolated from total PBMC using EasySep Myeloid DC enrichment kit (StemCell Technologies) according to the manufacturer's instructions. Samples were thawed, counted and resuspended in sort buffer at a concentration of 5 x 10^7 cells/ml. The cell suspension was transferred to a 5ml polystyrene FACS tube (BD Biosciences), and the FcR blocker was added (15µl/ml cells). The enrichment cocktail was added to the sample (50 µl/ml cells), and the mixture was incubated for 30 minutes at RT. Magnetic nanoparticles were then added (100µl/ml cells) and mixture was incubated for a 10 minutes at RT. The mixture was then topped up to 2.5ml with sort buffer and placed into the EasySep magnet for 5 minutes. Picking up the magnet, in one continuous movement, the supernatant (mDC enriched fraction)

was carefully poured into a new 5ml polystyrene tube. To ensure maximum purity, the new tube, containing the enriched mDC suspension was then placed in the magnet for further 5 minutes. Finally, the supernatant was poured off as before and the cells were counted.

2.4.3 Fluorescent Activated Cell Sorting (FACS) using FACSAriaIII (BD)

Antibody mixes were made up at the appropriate dilutions in sort buffer. CD3 depleted, or total PBMC/SFMC were resuspended in the antibody mix to a final concentration of $10x10^6$ cells per 100µl antibody mix, and incubated in the dark for 30 minutes at 4°C. The cells were washed in sort buffer and resuspended in sort buffer at ~$30x10^6$ cells per ml. The cell suspension was filtered through a 50µm filter before sorting. Sorted cells were collected into an Eppendorf containing RPMI supplemented with 20% FCS.

ILC were sorted as: CD45+lineage-CD127+CD161+

Naïve CD4+ T cells were sorted as: CD4+CD25-CD45RO-

mDC were sorted as: CD3-CD19-CD56-CD14-CD11c+

Purity of all samples was assessed post sort. For ILC purity, an aliquot of cells (~20µl) was taken from the collection Eppendorf tube and immediately run back through the FACSAriaIII to ensure no lineage+ cells had been collected. 1000 sorted ILC were also stained for ILC subtype specific markers (CRTH2, c-Kit and NKp44) to ensure that the sorting process did not affect expression compared to pre-sort samples.

2.5 Gene expression by quantitative polymerase chain reaction (qPCR)

2.5.1 RNA extraction

Total cellular RNA was extracted using the Arcturus Picopure RNA isolation kit (Life Technologies) in accordance with the manufacturer's instructions. RNA was extracted from sorted cell samples within 1 hour of sorting and stored in Picopure extraction buffer at -80°C until required. RNA was isolated from the frozen samples using spin columns, and DNA was removed by treatment with DNase as per the manufacturer's protocol. RNA absorbance at 260nm was measured to ascertain yield and purity using the NanoDrop 1000 Spectrophotometer and 260:280 ratio > 1.75 was deemed acceptable (Thermo Scientific) Since very low cell numbers of ILC were likely to be sorted from JIA PBMC and SFMC, the Picopure protocol was first validated to ensure RNA purity would be maintained for very low cell numbers. RNA was extracted from $1x10^5$, $0.5x10^5$, $2.5x10^4$ and $1.25x10^4$ total PBMC, yield and purity remained high at the lowest cell concentration (Figure 2.1). Since the number of ILC sorted from JIA PBMC and SFMC fell within the validated range, Picopure was considered a suitable method for RNA extraction.

2.5.2 cDNA synthesis

For initial analyses of ILC signature cytokines and transcription factors, RNA was converted to cDNA for use in subsequent qPCR reactions. Random hexamers (90ng/µl) (Invitrogen) were added to the isolated RNA as primers for cDNA synthesis. The mixture was incubated for 10 minutes at 37°C and then cooled on ice. 8.5µl of the master mix (4µl-5x First strand B°; 2µl-0.1M DTT; 1µl-10mM dNTPs; 1µl-RNase OUT; 0.5µl- Superscript RT) was added to the RNA/hexamer mix. This was then incubated for 10 minutes at RT, followed by 60 minutes at 42°C and finally 10 minutes at 72°C. 20µl H_2O was added and the resulting cDNA was stored at -80°C until use.

For qPCR analysis of mDC cytokine gene expression (*IL1B, IL6* and *IL23A)*, and expression of aldehyde dehydrogenase (ALDH) genes (*ALDH1A1, ALDH1A2* and *ALDH1A3)* and ILC expression retinoic acid receptor genes (*RARA, RARB* and *RARG*), RNA was extracted from FACS sorted cells using the Picopure protocol as described above. The RNA was subsequently converted to cDNA using iSCRIPT cDNA synthesis kit (Bio-Rad) according to the manufacturer's instructions. Following synthesis, cDNA was stored at -80°C until use.

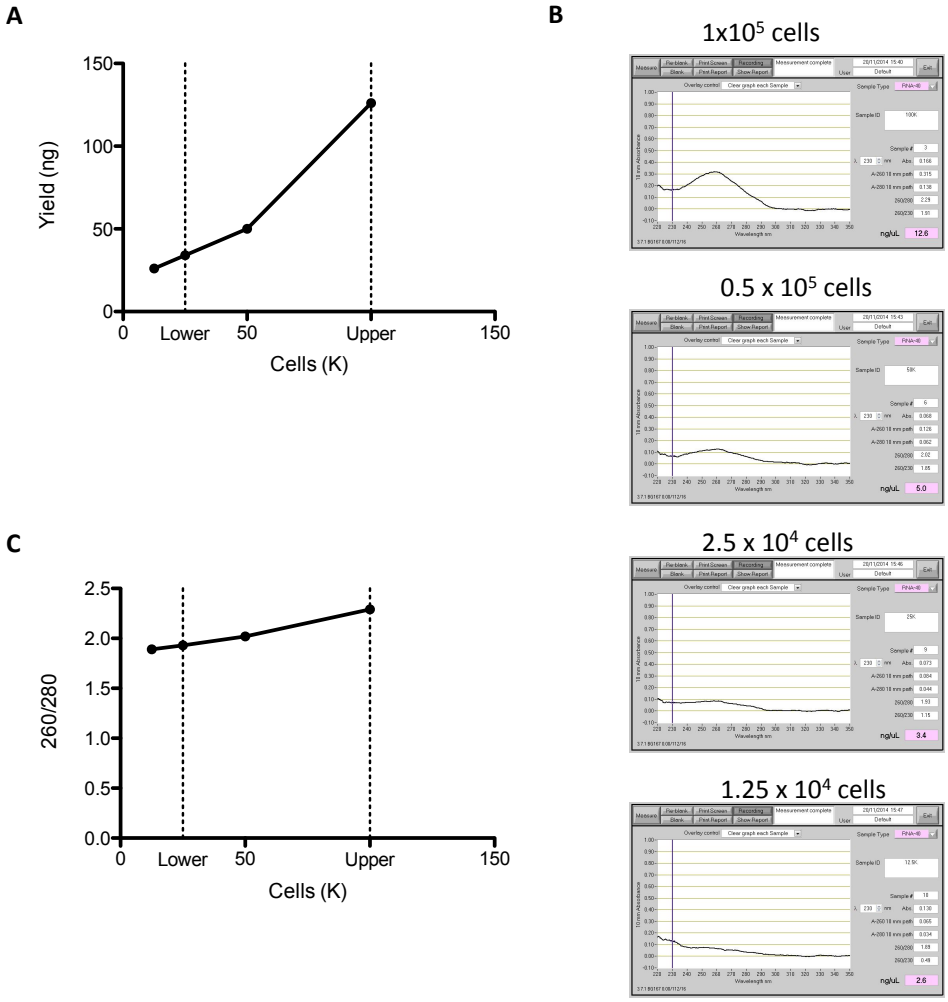


Figure 2.1 Optimisation of RNA extraction for very low cell numbers.

(A) RNA yield (ng) from samples of varying cell number ranging from 100K to 12.5K PBMC (B) NanoDrop analysis of RNA quality with decreasing cell number. (C) Summary of RNA quality achieved from varying cell number. Upper and lower bounds represent the highest and lowest number of ILCs FACS sorted.

2.5.3 qPCR

Quiagen Quantitect validated primers were used for some analyses. Alternatively, primers were chosen from publications and ordered specifically (see Table 2.3); specificity for the gene of interest was verified using primer BLAST software, custom made (Life Technologies) and validated for specificity through the analysis of melt curves generated following PCR amplification using SYBR Green Mastermix (BioRad) on the Rotor-gene 6000 Corbert thermocycler (Qiagen). Each primer generated a single peak and the predicted size was confirmed by electrophoresis on a 1% agarose get in TRIS-EDTA buffer (Figure 2.2).

To make the gel, 1g of pure agarose was added to 100ml of 1xTRIS-EDTA buffer and melted in a microwave for 3 minutes on high setting. The high temperature allowed the agarose to dissolve, and the mixture was left for 5 minutes to cool. Once cooled, 5µl GelRed (Biotum) was added and the mixture was carefully poured into an appropriate gel tray. Samples were routinely prepared as follows:

Samples: 2µl test DNA + 1µl loading buffer II (New England Biolabs) + 3µl nuclease free water. 5µl 100bp ladder (New England Biolabs) + 1µl loading bufferII (Ambion) was run in parallel at 150V for 30 minutes.

The reaction mix consisted of:
10µL 2XSYBR Green Mastermix
6 µL DNase-free water,
1 µL forward primer at 10µM (500 nM final concentration)
1 µL reverse primer at 10µM (500 nM final concentration)
2µl cDNA template or H_20 for no template control (NTC)
20µL Final Volume

The cycling conditions were:

95°C 5 mins

Followed by 40 cycles of:

95°C 30s

60°C 30s

72°C 30s

For the analysis of signature ILC cytokines and transcription factors, standard curves were generated from polarized T-cells, and used to quantify gene expression. For the analysis of mDC cytokines, mDC were isolated from healthy control PBMC and subsequently stimulated with LPS (100ng/ml for 15 hours). Post-stimulation, increased cytokine expression allowed the extraction of cytokine RNA and cDNA. This cDNA served as a template for the generation of cytokine-specific standard curve(s). To control for inter-PCR run variation, one point on the standard curve was included in each PCR run in order to calibrate the imported standard curve. This gave a value in arbitrary units (AU) for expression of each gene. Expression was calculated relative to housekeeping gene *ACTB* (beta-actin) by dividing through the 'gene of interest' expression in AU by *ACTB* expression.

Gene	Primer Sequences 5'-3'		Annealing Temp (°C)	Amplicon Size (bp)	Efficiency (%)	Supplier
ACTB	F	AGATGACCCAGATCATGTTTGAG	60	187	91	Life Technologies
	R	AGGTCCAGACGCAGGATG				
TBX21	F	CCCCAAGGAATTGACAGTTG	60	337	92	Life Technologies
	R	GGGAAACTAAAGCTCACAAAC				
IFNG	F	TGACCAGAGCATCCAAAAGA	60	236	95	Life Technologies
	R	CTCTTCGACCTCGAAACAGC				
GATA3	F	ACCACAACCACACTCTGGAGGA	60	132	102	Life Technologies
	R	TCGGTTTCTGGTCTGGATGCCT				
IL13	F	ATTGCTCTCACTTGCCTTGG	60	152	81	Life Technologies
	R	GTCAGGTTGATGCTCCATACC				
RORC	F	AATCTGGAGCTGGCCTTTCA	60	129	98	Life Technologies
	R	CTGGAAGATCTGCAGCCTTT				
AHR	F	CTTAGGCTCAGCGTCAGTTA	60	130	101	Life Technologies
	R	GTAAGTTCAGGCCTTCTCTG				
IL17A	F	AATCTCCACCGCAATGAGGA	60	95	97	Life Technologies
	R	ACGTTCCCATCAGCGTTGA				
IL22	F	CCCATCAGCTCCCA	60	257	88	Life Technologies

		CTGC				
	R	GGCACCA CCTCCTG CATATA				
RARA	n/a		60	148	97	Qiagen
RARB	n/a		60	118	n/a	Qiagen
RARG	n/a		60	147	n/a	Qiagen
IL6	n/a		60	107	81	Qiagen
IL23A	n/a		60	149	71	Qiagen
IL1B	n/a		60	117	85	Qiagen
ALDH1A1	n/a		60	97	73	Qiagen
ALDH1A2	n/a		60	80	111	Qiagen
ALDH1A3	n/a		60	125	n/a	Qiagen
GAPDH	n/a		60	119	93	Qiagen

Table 2.3 Primer pairs utilized in qPCR assays

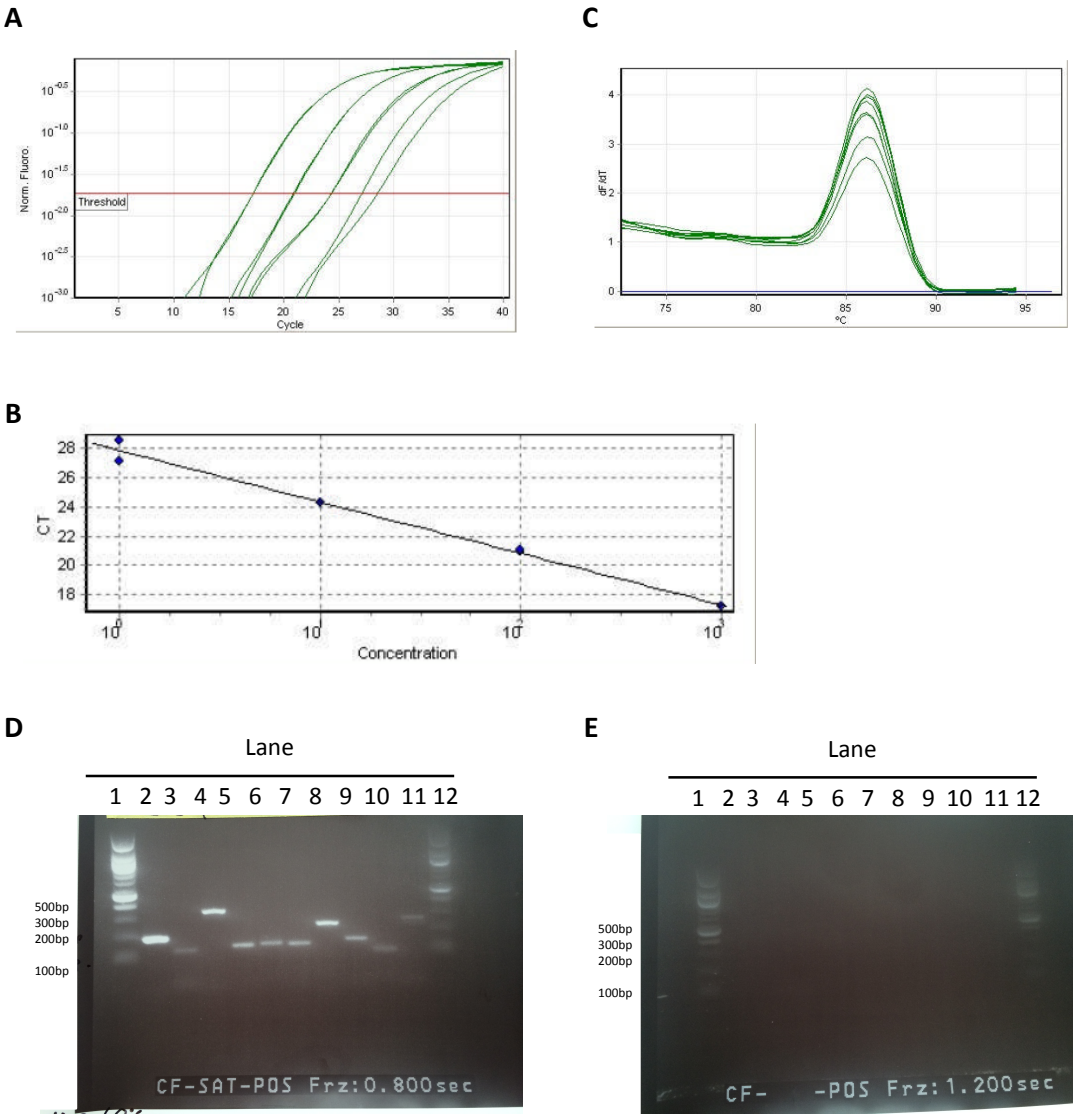


Figure 2.2 Optimisation of custom made primers for ILC qPCR

Example of optimisation procedure for *IFNG* (A) Template titration; each curve represents PCR reaction with defined template (B) Standard curve generated from titration data shown in A (C) Representative melt curve. (D) Product size confirmation by agarose- gel electrophoresis (E) Reactions run with no template. Gel band sizes confirmed using ladder (lanes 1 and 12 on each gel). Lanes show amplification products for: Lane 2- *ACTB*; lane 3- *GAPDH*; lane 4-*TBX21* (Tbet); lane 5- *RORC*; lane 6- *GATA3*; lane 7- *AHR*; lane 8- *IFNG* (IFNγ); lane 9 – *IL-13*; lane 10 – *IL-17A*; lane 11- *IL-22*.

2.6 Cell culture

2.6.1 T cell polarization

FACS sorted naïve CD4+ T cells (CD4+CD21-CD45RO-) from healthy adult PBMC (sorted sample >98% pure) were seeded onto a 96-well round bottom plate coated with 1µg/ml anti-CD3 (R&D systems) and 5µg/ml anti-CD28 (BD Pharmingen) and incubated for 4 days (37°C and 5% CO_2) with skewing cytokines. For skewing towards different T cell subsets, the cultures were supplemented with the following recombinant human cytokines (Peprotech Ltd)
Th1 – IL-12 (10ng/ml final concentration)
Th2 – IL-4 (50ng/ml final concentration)
Th17 – TGF-β (10ng/ml final concentration), IL-6 (20ng/ml final concentration), IL-1β (10ng/ml final concentration), IL-23 (100ng/ml final concentration)

Effective polarization of T cell subsets was confirmed by flow cytometry analysis and verified by amplification of transcripts for canonical transcription factors and cytokines following the protocol detailed above and normalized to *ACTB* using delta CT (Figure 2.3).

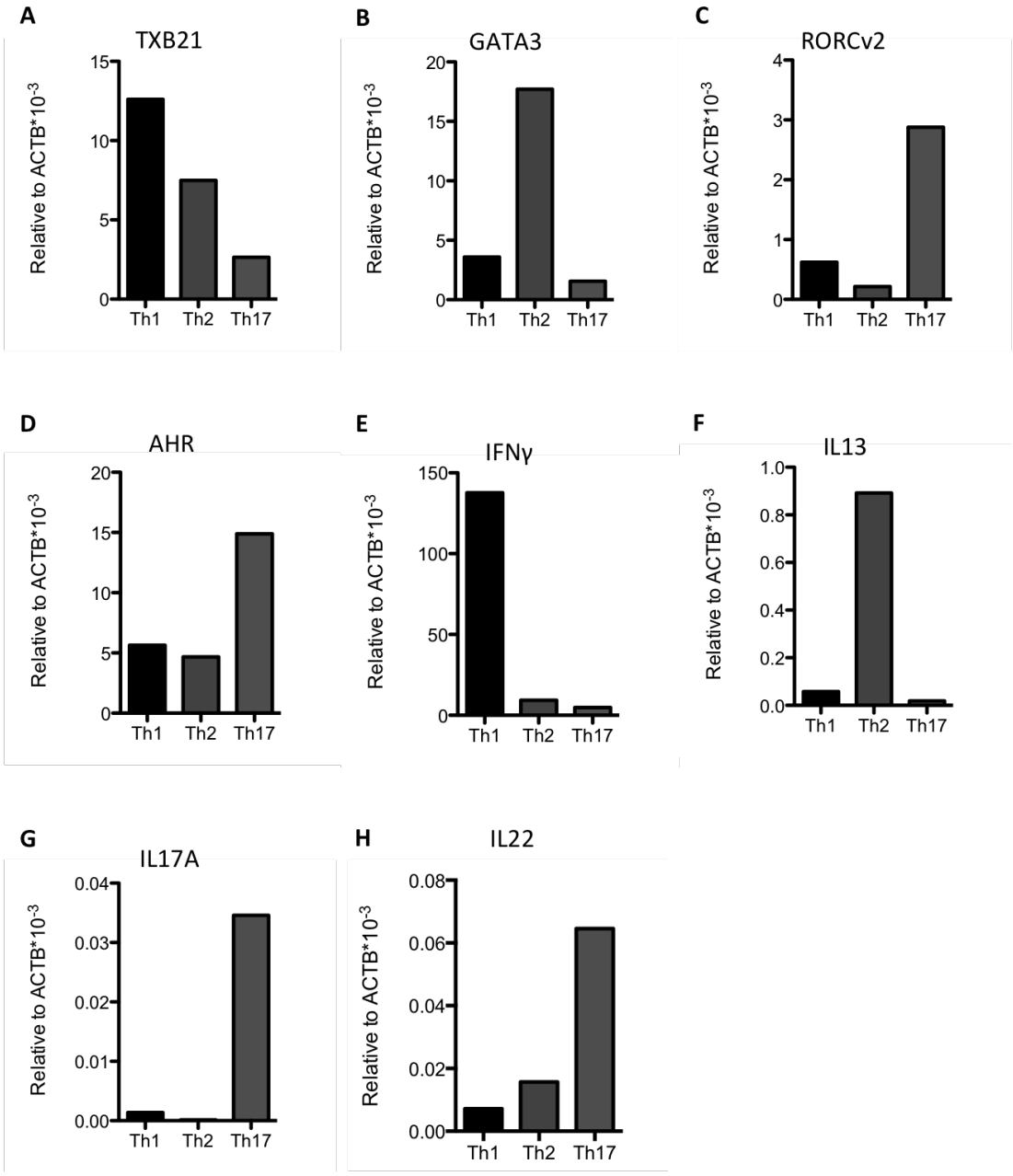


Figure 2.3 Relative expression of mRNA transcripts for selected signature genes in Th1, Th2 and Th17 polarised cells.

RNA and cDNA were prepared from 4-day polarised CD4+CD25-CD45RO- FACS sorted T-cells. Cells were cultured in IL-12 for Th1, IL-4 for Th2 and TGFβ, IL-6, IL-1β and IL-23 for Th17 polarising conditions. Relative expression in Th1 polarised, Th2 polarised and Th17 polarised cells as shown, of mRNA for (A) *TXB21* (B) *GATA3* (C) *RORC* (D) *AHR* (E) *IFNG* (F) *IL-13* (G) *IL-17A* and (H) *IL-22*. Each bar represents the mean value of duplicate wells run and expressed relative to the *ACTB* control.

2.6.2 LPS mediated mDC stimulation

FACS sorted mDC (CD14-CD11c+) ($1x10^5$cells/well) were cultured overnight in 10% RPMI supplemented with LPS (100ng/ml, 15 hours). After overnight culture, cells were washed with 10% RPMI and RNA was extracted following the Picopure protocol as described above.

2.6.3 ILC activation culture for cytokine analysis

FACS sorted ILC were seeded into a 96 well plate (2000 cells/well) in 10%RPMI medium, supplemented with recombinant human IL-2 (0.5ng/ml) (Peprotech) plus skewing cytokines or 10-50% SF serum. For specific activation of different ILC subsets, the cultures were additionally supplemented with the following cytokines (all 50ng/ml final concentration):

ILC1 – IL-12, IL-18
ILC2 – Thymic stromal lymphopoietin (TSLP), IL-25, IL-33
ILC3 – IL-23, IL-1β

Following 4 days incubation (37°C and 5% CO_2), the cells were washed once with 10% RPMI and cytokines were stained as previously described.

2.6.4 ILC skewing assays

FACS sorted ILC were seeded into a 96 well plate in Yessel's medium supplemented with 1% human AB serum (Gemino Bio-products) and IL-2 (50ng/ml) with IL-6 (50ng/ml), SF (50%) or all-trans retinoic acid (RA) (1µM; Sigma-Aldrich) in the presence or absence of the subtype specific skewing cytokines (all 50ng/ml final concentration) described above. Following 7 days incubation (37°C and 5% CO_2), the cells were washed once with 10%RPMI and ILC skewing was assessed by surface marker expression and cytokine production.

2.7 Aldefluor Assay

Retinoic acid (RA) production by PMBC and SFMC was assessed using the Aldefluor kit (StemCell Technologies) as per the manufacturer's instructions. Frozen cells were thawed and resuspended in Aldefluor Assay Buffer (StemCell Technologies) at a concentration of 1 x 10^6 cells/test and the sample was split equally between two 1.5ml Eppendorf tubes. To the control tube, 10µl of Aldefluor diethylaminobenzaldehyde (DEAB) reagent was added to block binding of the aldefluor reagent to ALDH enzymes within the cells, and the solution was mixed using a vortex. 5µl of the activated Aldefluor reagent was added to each test and control sample. The samples were immediately vortexed to mix, and incubated for 45 minutes at 37°C. Next, the samples were centrifuged to pellet the cells (300g for 5 minutes) and the supernatant was carefully removed. The pellet was finally resuspended in Aldefluor buffer and subsequent surface staining was carried out in Aldefluor buffer instead of usual FACS buffer.

2.8 Statistical analysis

Statistical analysis was carried out on GraphPad Prism 6. Mean or median values are shown on summary plots, and error bars represent standard error of the mean (SEM). Data were considered non-Gaussian and therefore non-parametric statistical tests were employed. For comparison of two groups Mann-Whitney U Tests and for multiple comparison testing, Kruskal-Wallis tests were used with Dunn's multiple comparisons tests. Spearman's correlation was used for analysis of correlations. P values of less than 0.05 were considered significant and indicated on graphs as follows: *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$.

3 : Defining the T cell signature in ERA JIA

3.1 Introduction

Given the distinct features and genetic associations of the ERA JIA subtype of JIA compared to the other forms of childhood arthritis, discussed in Chapter 1, it is likely that distinct mechanisms may play a role in the pathogenesis of this form of arthritis. Investigation of the pathogenesis of ERA JIA to date has been limited and largely restricted to Asian populations, where the ERA JIA subtype of JIA is more prevalent (Agarwal et al., 2008a, Mahendra et al., 2009, Myles and Aggarwal, 2011, Myles et al., 2012, Gaur et al., 2015, Gaur et al., 2017). These studies have primarily focused on the involvement of innate immune cells in particular monocytes and monocyte derived inflammatory mediators (Saxena et al., 2005, Agarwal et al., 2009, Myles and Aggarwal, 2011, Myles et al., 2012). However, initial studies allude to IL-17 production from NK and T cells in ERA JIA patients, and a role for IL-17 in disease pathogenesis has been suggested, although further analysis is warranted (Mahendra et al., 2009, Gaur et al., 2015).

It is well known that T cell immunity plays a key role in the pathogenesis of inflammatory arthritis in adults and children (Froland et al., 1973, Nistala and Wedderburn, 2009, Nistala et al., 2010b, Omoyinmi et al., 2012, Shabgah et al., 2014). While relatively little is known about the T cell involvement in ERA JIA, biological similarities with other JIA subtypes, or adult spondyloarthropathies have been hypothesized. Published reports of T cell derived- IL-17 in ankylosing spondylitis (AS), and the clear genetic and clinical parallels between adult AS and childhood onset ERA JIA strengthen the hypothesis of potential IL-17 involvement in ERA JIA (Schlosstein et al., 1973, Newport et al., 2007, Wendling et al., 2007, Shen et al., 2009, Chen et al., 2014a). Furthermore, the immunophenotype of oligoarticular arthritis has been well investigated and CD4 T cells producing inflammatory IL-17 have been implicated in disease (Nistala and Wedderburn, 2009, Nistala et al., 2010a, Spreafico et al., 2016). It is now well established that CD8 and γδ T cell populations can also produce IL-17 (Walker et al., 2012, Kenna et al., 2012). At present, IL-17-production from these T cell populations has not been thoroughly investigated in JIA.

Regulatory T cells (Treg) have been extensively studied in JIA, where they are shown to have a reciprocal relationship to Th17 cells in oligoarthritis (Nistala and Wedderburn, 2009, Olivito et al., 2009). More recently, the presence of cytokine producing Treg, which express killer cell lectin-like receptor, CD161 have been described at the inflamed site (Pesenacker et al., 2013). Interestingly, these CD161+Treg express RORC and share significant transcriptional homology with Th17 cells (Duurland et al., 2017a). Neither Treg, nor CD161+Treg have been examined in the synovial fluid from ERA JIA patients from UK cohorts.

Informed by these preliminary studies in ERA JIA, as well as the known involvement of the IL-23/IL-17 pathway in AS and other subtypes of JIA, this chapter aimed to investigate the T cell profile in the blood and joints of ERA JIA patients with a focus on IL-17 and associated cytokines: IFNγ, IL-22 and GM-CSF. It was hypothesised that poly-functional IL-17-producing T cells would be expanded in the synovial fluid of ERA JIA patients in all T cell compartments and would be associated with disease severity. In parallel, an expansion of CD161+Treg at the inflamed site in ERA JIA patients was also predicted. These aims were addressed by:

1. Performing detailed immune cell phenotyping in the PBMC and SFMC from JIA patients including a significant cohort of patients with ERA JIA.
2. Investigating cytokine production, and poly-functionality of CD4, CD8 and CD4-CD8- T cells from the blood and synovial fluid from JIA patients,
3. Probing for associations between IL-17-positive (IL-17+) T cell populations and clinical measures of disease severity in ERA JIA.
4. Analysing Treg populations in the blood and synovial fluid from ERA JIA patients.

3.2 Results

3.2.1 mDC and CD8+ T cells are enriched in the SFMC of ERA JIA patients.

While there are significant published data describing the immune phenotype in other subtypes of JIA, there has been limited investigation into the immunophenotype in ERA JIA. In order to gain an overview of the immune cells at the inflamed site in ERA JIA, multicolour flow cytometry analysis of immune cells from within the PBMC and SFMC of ERA JIA patients was carried out and results were compared to data from the PBMC and SFMC of other JIA subtypes (oligoarticular, polyarticular and psoriatic) and adult and child healthy control PBMC.

Following the exclusion of dead cells and doublets (Figure 3.1A), the proportions of various mononuclear cell types (monocytes, mDC, B cells, NK cells and T cells) were enumerated within the scatter gate in PBMC and SFMC from patients (n=16, n=39) and PBMC of adult (n=12) and child (n=3) healthy controls. Figure 3.1 shows the gating strategy used for the identification of: monocytes (CD14+); mDC (CD14-CD11c+); B cells (CD3-CD19+); NK cells (CD3-CD56+); and T cell subpopulations (CD3+CD4+, CD3+CD8+ and CD3+CD4-CD8-γδTCR+) in PBMC (Figure 3.1B) and SFMC (Figure 3.1C) from a representative ERA JIA patient. Mononuclear cells from synovial fluid are typically larger and more granular than cells from PBMC according to forward and side scatter (SSC-A) (top left panel from Figures 3.1B and 3.1C).

The summary data for these analyses are detailed in Figure 3.2. While significant differences were seen between PBMC and SFMC from JIA patients, no significant differences were noted in the immune cell populations as proportion of total live cells, within the PBMC between JIA subtypes, or within SFMC between JIA subtypes (Figure 3.2A-F). Proportions of monocytes, NK cells and CD4 T cells were not significantly different between PBMC and SFMC from ERA JIA patients (Figures 3.2A, 3.2D and 3.2E). mDC were significantly enriched ($p<0.0001$) in the SFMC from ERA JIA patients compared to paired

PBMC (Figure 3.2B). The role of enriched SFMC mDC is explored in greater detail in Chapter 5 of this thesis. CD8 T cells were also significantly enriched in ERA JIA SFMC compared to the PBMC compartment ($p=0.048$; Figure 3.2F). This enrichment was further highlighted when the ratio of CD4:CD8 T cells was analysed in the PBMC from aHC and cHC and the PBMC and SFMC from all JIA subtypes and from ERA JIA patients. A significant decrease in the T cell ratio was seen between PBMC and SFMC in JIA patients, with a median CD4:CD8 ratio of 1.02 in the SFMC compared to 1.60 in paired PBMC from ERA JIA patients ($p<0.001$) (Figure 3.2H). Conversely, there was a significant reduction of B cells in the SFMC compared to PBMC of ERA JIA patients ($p<0.0001$) (Figure 3.2C). No significant difference was seen in the size of the γδ T cell population between PBMC and SFMC from ERA JIA patients, however ANOVA analysis revealed γδ T cells were significantly enriched in the SFMC compared to PBMC in other JIA subtypes combined ($p<0.01$) (Figure 3.2G).

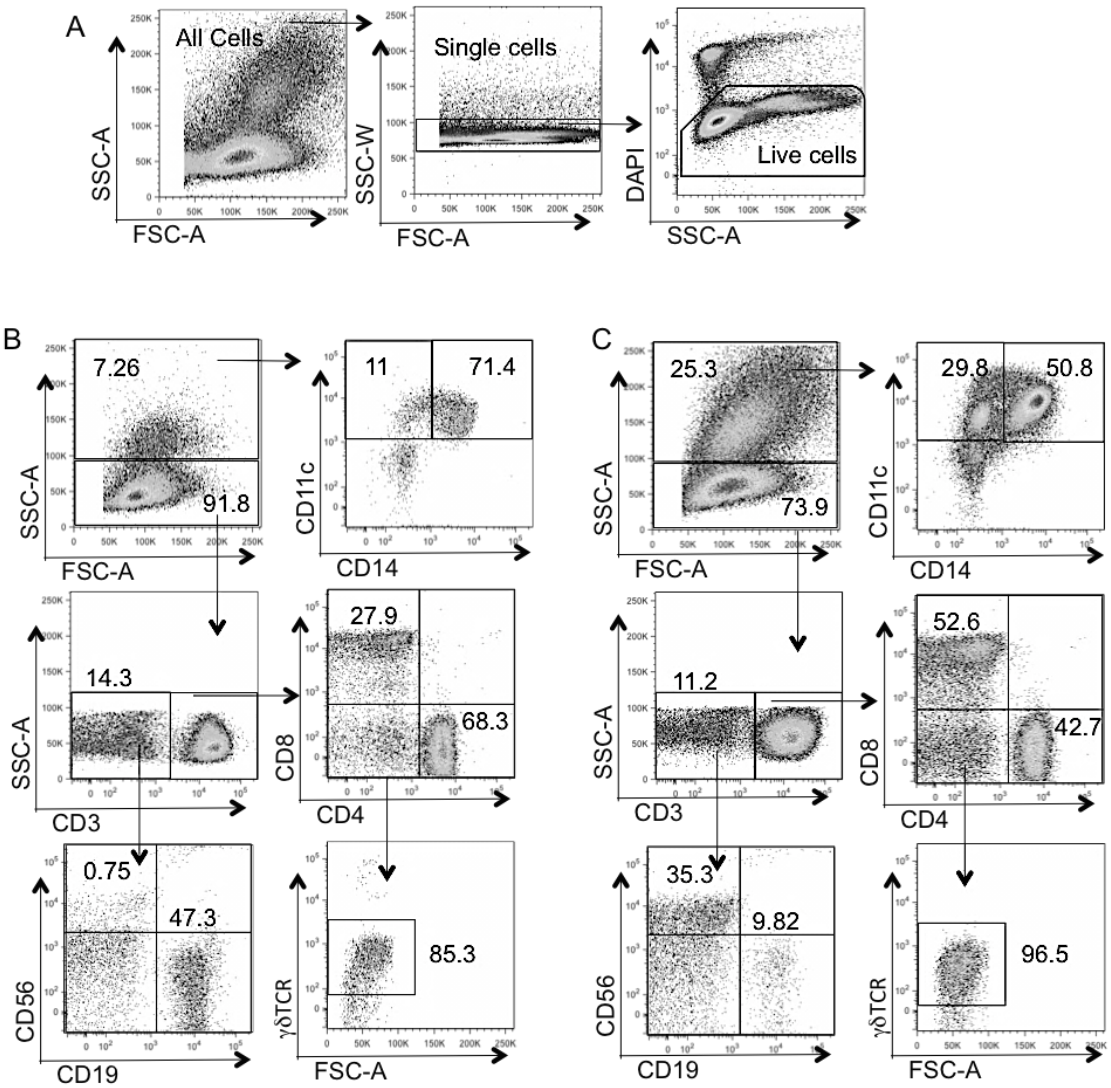


Figure 3.1 Gating strategy for the identification of immune cell types from blood and synovial fluid mononuclear cells from JIA patients and controls.

Representative gating strategy for (A) The exclusion of doublets and dead cells. Data generated from 1 adult control PBMC. (B and C) Enumeration of T cells (CD3+CD4+, CD3+CD8+ and CD3+CD4-CD8-γδTCR+), B cells (CD3-CD19+), NK cells (CD3-CD56+), monocytes (CD14+) and mDC (CD14-CD11c+) after exclusion of dead cells and doublets, from (B) PBMC and (C) SFMC from a patient with ERA JIA. Black boxes represent gates. The numbers show % of events in a specific gate.

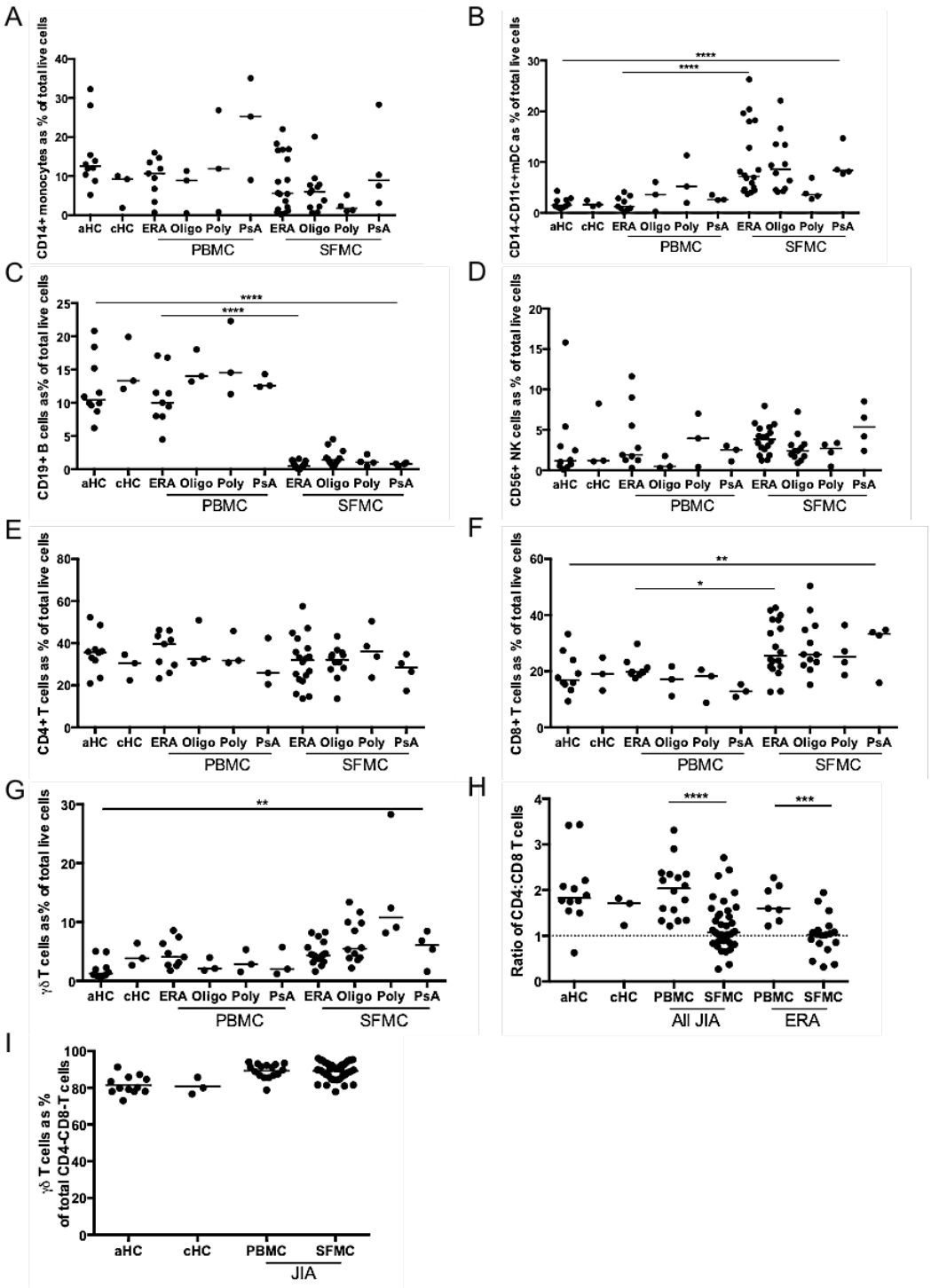


Figure 3.2 mDC and CD8+ T cells are enriched in the SFMC of ERA JIA patients.

(A-G) Summary plots showing enumeration of immune cell types using gates as shown in Figure 3.1 within PBMC of healthy adult (aHC) and child controls (cHC)(n=10, n=3 respectively) and PBMC and SFMC from ERA-JIA (n=9, n=18), oligo-JIA (n=3, n=12) poly-JIA (n=3, n=4) and PsA-JIA patients (n=3, n=4), shown as a percentage of total live mononuclear cells for (A) monocytes (CD14+); (B) mDC (CD14-CD11c+); (C) B cells (CD3-CD19+);(D) NK cells (CD3-CD56+); (E) CD4 T cells (CD3+CD4+);(F) CD8 T cells (CD3+CD8+) and (G) γδ T cells (CD3+CD4-CD8-γδTCR+). (H) Ratio of CD4:CD8 T cells in the PBMC and SFMC of JIA and ERA JIA patients and healthy PBMC. (I) Summary plot showing γδ T cells as a % of CD3+CD4-CD8- cells. Bars represent median values. Statistical analysis by Kruskal Wallis and Mann-Whitney. *p<0.05**p<0.01***p<0.001****p<0.0001.

3.2.2 Cytokine-producing T cells are enriched in the SFMC of ERA JIA patients.

Cytokine producing T cells, and in particular IL-17- producing CD4 T cells have been reported in the SFMC from oligo-JIA patients and are implicated in disease pathogenesis (Spreafico et al., 2016). While there are only limited data available on immune pathogenesis of ERA JIA, some studies have suggested a role for IL-17 production by T cells (Agarwal et al., 2008b, Mahendra et al., 2009) To further our understanding of the role of T-cell derived cytokines in ERA JIA, IL-17, IFNγ, and IL-22 production from T cell subpopulations was analysed (in PBMC and SFMC samples) from ERA JIA, other JIA subtypes (oligo-JIA, poly-JIA and PsA-JIA) and PBMC from aHC and cHC by flow cytometry following 4 hour stimulation with PMA and Ionomycin in the presence of Brefeldin A. Due to time restrictions, T-cell derived GM-CSF production was only investigated from PBMC (ERA JIA and aHC) and SFMC from ERA JIA patients. Cytokine production was analysed from CD4, CD8 and CD4-CD8- T cell (CD3+) subpopulations (gating as shown in Figure 3.1B and 3.1C). γδ T cells represented >80% of the CD3+CD4-CD8- cell population in all PBMC and SFMC samples analysed (Figure 3.2I). For the purpose of these studies, CD4-CD8- T cells were therefore deemed to be representative of γδ T cells.

CD4 T cells from PBMC and SFMC of JIA patients and healthy controls were analysed for production of IFNγ (Figure 3.3A), IL-17 (Figure 3.3B), IL-22 (Figure 3.3C) and GM-CSF (Figure 3.3D). Approximately 6-10% of CD4 T cells from the PBMC compartment of aHC (median=9.16 IQR=4.14-14.33), cHC (median=8.57 IQR=6.57-10.18) and ERA JIA patients (median=8.22 IQR=4.61-10.88) were IFNγ producers. In contrast, typically >40% of SFMC CD4 T cells were IFNγ+ in the SFMC (median= 40.90 IQR=38.58-62/08) ($p<0.0001$, Figure 3.3.E). Similarly, IL-17 and GM-CSF-production was also significantly increased in CD4 T cells from the SFMC from ERA JIA patients compared to paired PBMC (for IL-17: ERA JIA PBMC median= 0.62 IQR=0.40-1.57, ERA JIA SFMC median= 3.79 IQR=1.77-5.99; for GM-CSF: ERA JIA PBMC median= 4.55 IQR=3.42-6.93, ERA JIA SFMC median= 12.09 IQR=9.26-15.33) ($p=0.001$ and $p=0.0022$ for IL-17 and GM-CSF respectively; Figures 3.3F and 3.3H).

Interestingly, IL-22 + CD4 T cells were not significantly different between PBMC and SFMC from ERA JIA patients (Figure 3.3G).

When comparing cytokine production from CD4 T cells from the SFMC across JIA subtypes, significantly more IL-17+ cells were observed in ERA JIA patients compared to oligo-JIA ($p=0.0054$; Figure 3.3F), in whom elevated levels of IL-17 have previously been reported (Spreafico et al., 2016). Additionally, there were increased IL-17+ CD4 T cells in the PBMC of ERA JIA compared to oligo-JIA patients, although this did not reach statistical significance ($p=0.057$) (Figure 3.3F) perhaps due to small numbers of samples analysed. Unlike IL-17+ CD4 T cells, the number of IL-22 + CD4 T cells in the PBMC from ERA JIA patients when compared to oligo-JIA patients, did show a statistical increase ($p=0.0055$; Figure 3.3G).

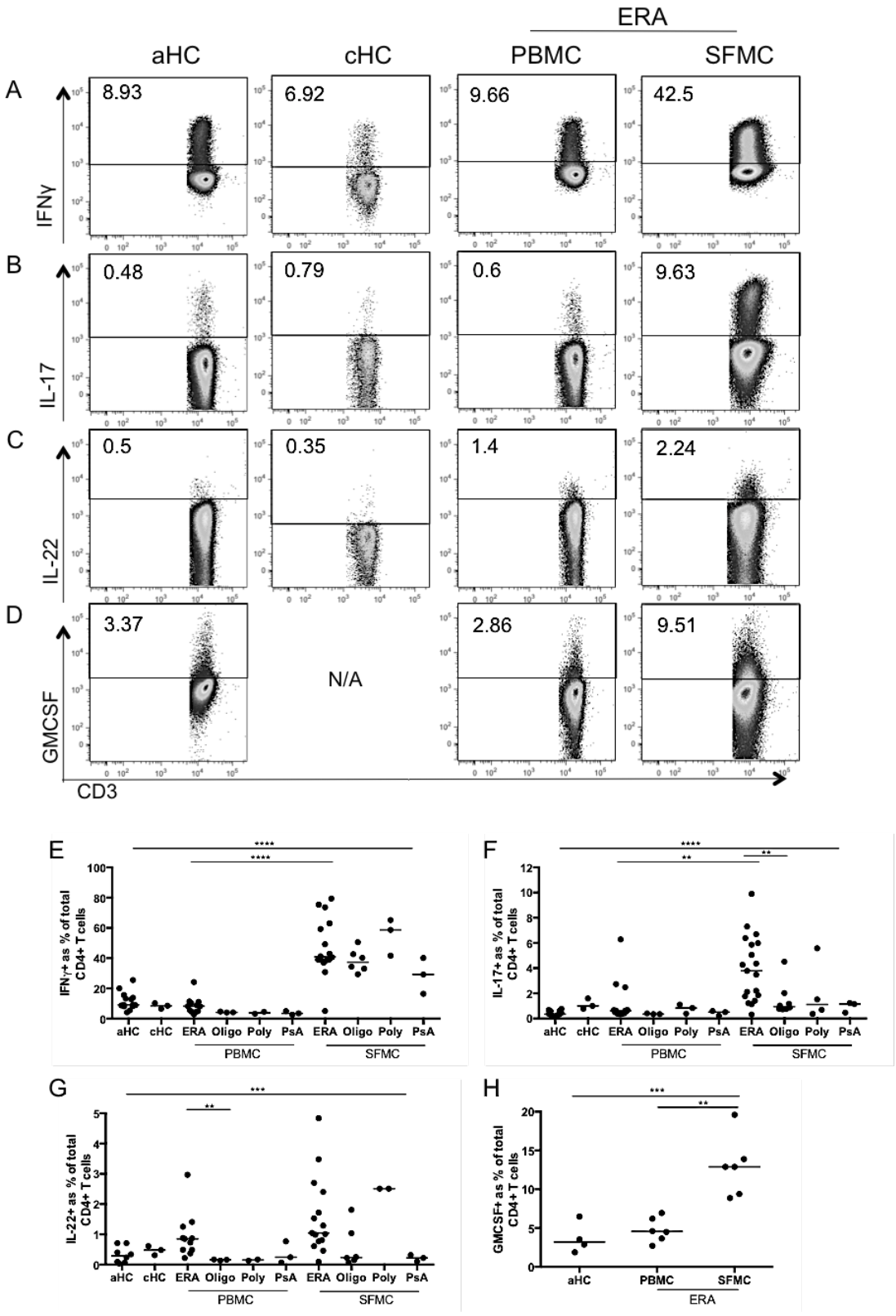

ERA
aHC
cHC
PBMC
SFMC
A
IFNγ
8.93
6.92
9.66
42.5
B
IL-17
0.48
0.79
0.6
9.63
C
IL-22
0.5
0.35
1.4
2.24
D
GMCSF
3.37
N/A
2.86
9.51
CD3
E
IFNγ+ as % of total CD4+ T cells
F
IL-17+ as % of total CD4+ T cells
G
IL-22+ as % of total CD4+ T cells
H
GMCSF+ as % of total CD4+ T cells
aHC cHC ERA Oligo Poly PsA ERA Oligo Poly PsA
PBMC
SFMC

Figure 3.3 Cytokine-producing CD4 T cells are enriched in the SFMC of ERA JIA patients.

Cytokine (IFNγ, IL17, IL-22 and GM-CSF) production by CD4 T cells (CD3+) was analysed by flow cytometry following 4 hours stimulation with PMA and Ionomycin in the presence of Brefeldin A, within PBMC from aHC and cHC (n=12, n=3 respectively) and PBMC and SFMC from ERA-JIA (n=13, n=18), oligo-JIA (n=3, n=8) poly-JIA (n=3, n=4) and PsA-JIA patients (n=3, n=3). (A-D) Representative plots showing the gating strategy for identification of cytokine positive CD4 T cells from aHC, cHC and PBMC and SFMC from an ERA JIA patient for (A) IFNγ; (B) IL-17A; (C) IL-22 and (D) GM-CSF. (E-H) Summary plots of cytokine positive CD4 T cells for (E) IFNγ; (F) IL-17A; (G) IL-22 and (H) GM-CSF. GM-CSF was analysed only in the ERA group, n=6 for both PBMC ad SFMC. Bars represent median values. Statistical analysis by Kruskal Wallis and Mann-Whitney. *p<0.05**p<0.01***p<0.001****p<0.0001.

When CD8 cells were analysed for production of the same cytokines, similar trends were seen (Figure 3.4A-D). Significantly more CD8 T cells + for IFNγ, IL-17 and GM-CSF were found in the SFMC of ERA JIA patients compared to paired PBMC (for IFNγ: ERA JIA PBMC median= 18.20 IQR=10.05-27.18, ERA JIA SFMC median= 50.80 IQR=46.03-58.63; for IL-17: ERA JIA PBMC median= 0.31 IQR=0.20-0.45, ERA JIA SFMC median= 0.79 IQR=0.46-1.63; for GM-CSF: ERA JIA PBMC median= 3.06 IQR=2.83-3.22, ERA JIA SFMC median= 5.42 IQR=3.93-7.23) (p<0.0001, p=0.0003 and p=0.0065 respectively; Figures 3.4E, 3.4F and 3.4H). Again, significantly higher IL-17 production was seen in CD8 T cells in the SFMC of ERA JIA patients compared to oligo-JIA patients (ERA JIA median= 0.79 IQR=0.46-1.63; oligo JIA median= 0.18 IQR=0.09-0.90) (p=0.0093; Figure 3.4F). Increased IL-17+ CD8 T cells were seen in ERA JIA PBMC compared to oligo-JIA patients (ERA JIA median= 0.31 IQR=0.20-0.45; oligo JIA median= 0.11 IQR=0.11-0.12) (p=0.047) (Figure 3.4F). The abundance of IL-22 + CD8 T cells was not significantly different between PBMC and SFMC from ERA JIA patients (Figure 3.4G).

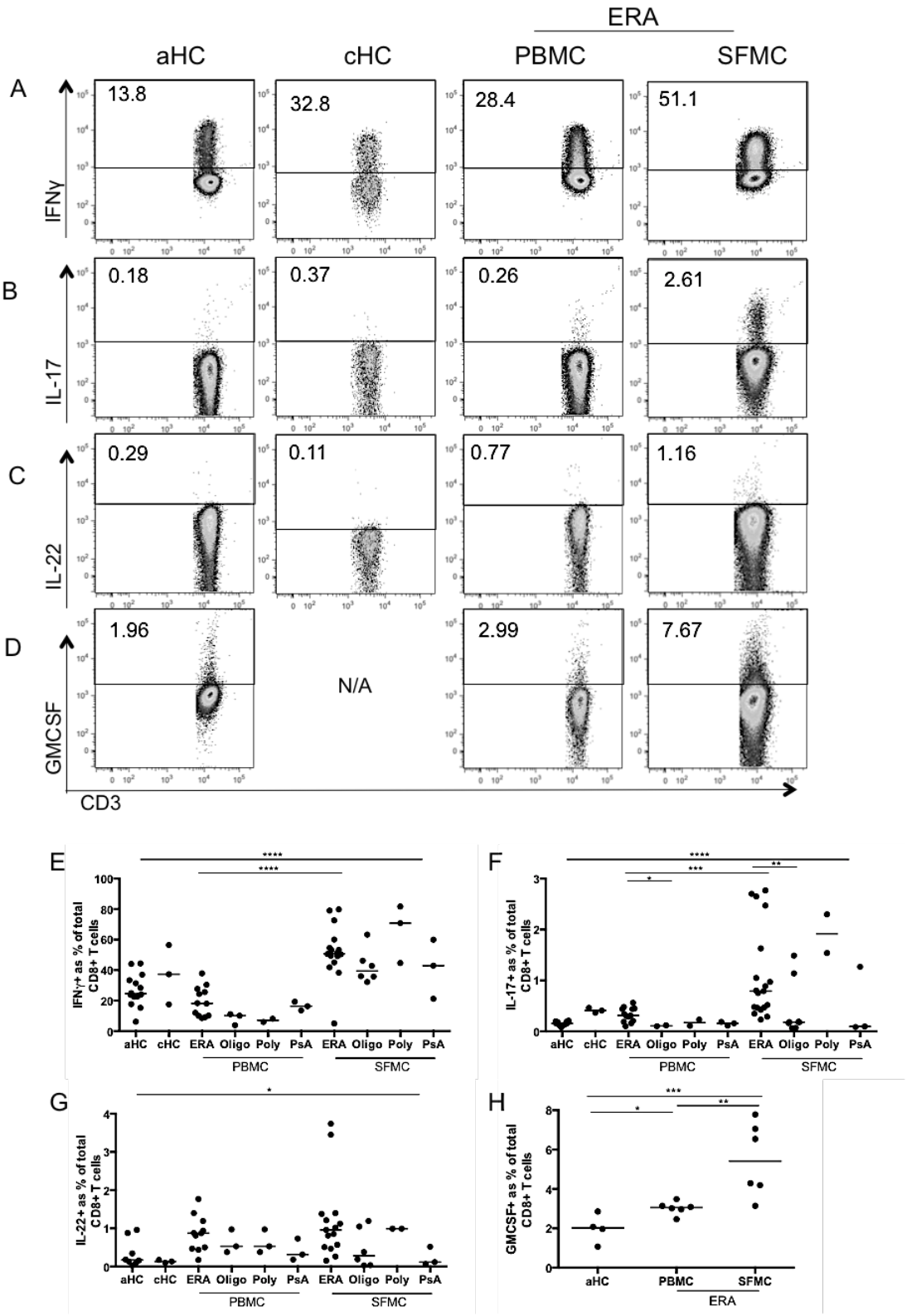
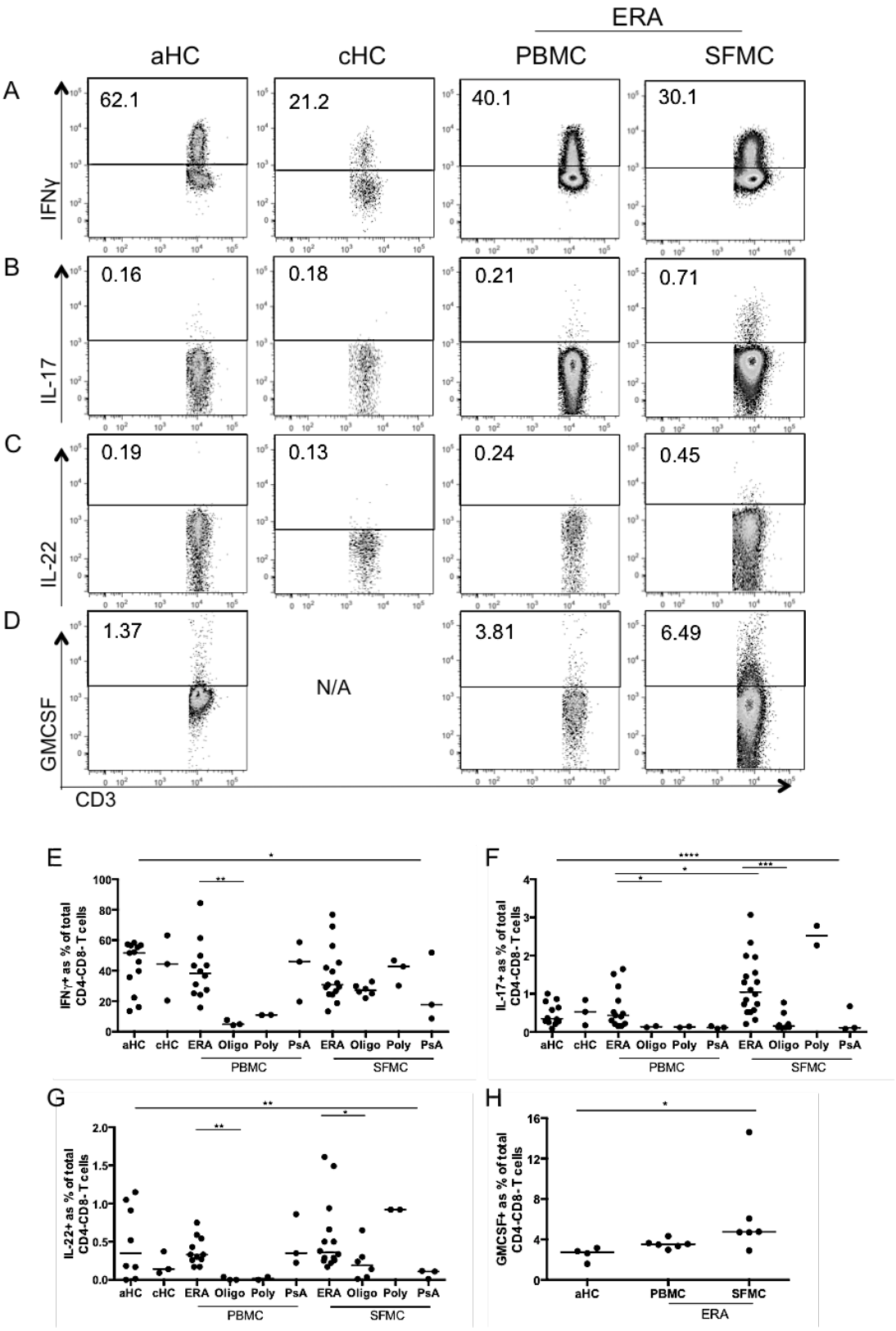

ERA
aHC
cHC
PBMC
SFMC
A
IFNγ
13.8
32.8
28.4
51.1
B
IL-17
0.18
0.37
0.26
2.61
C
IL-22
0.29
0.11
0.77
1.16
D
GMCSF
1.96
N/A
2.99
7.67
CD3
E
IFNγ+ as % of total CD8+ T cells
aHC cHC ERA Oligo Poly PsA ERA Oligo Poly PsA
PBMC
SFMC
F
IL-17+ as % of total CD8+ T cells
G
IL-22+ as % of total CD8+ T cells
H
GMCSF+ as % of total CD8+ T cells
aHC
PBMC
SFMC
ERA

Figure 3.4 Cytokine-producing CD8 T cells are enriched in the SFMC of ERA JIA patients.

Cytokine production by CD8 T cells (CD3+) was analysed by flow cytometry following 4 hours stimulation with PMA and Ionomycin in the presence of Brefeldin A, within PBMC form aHC and cHC (n=12, n=3 respectively) and PBMC and SFMC from ERA-JIA (n=13, n=18), oligo-JIA (n=3, n=8) poly-JIA (n=3, n=4) and PsA-JIA patients (n=3, n=3). (A-D) Representative plots showing the gating strategy for identification of cytokine positive CD8 T cells from aHC, cHC and PBMC and SFMC from an ERA JIA patient for (A) IFNγ; (B) IL-17A; (C) IL-22 and (D) GM-CSF. (E-H) Summary plots of cytokine positive CD8 T cells for (E) IFNγ; (F) IL-17A; (G) IL-22 and (H) GM-CSF. GM-CSF was analysed only in the ERA group, n=6 for both PBMC ad SFMC. Bars represent median values. Statistical analysis by Kruskal Wallis and Mann-Whitney. *p<0.05**p<0.01***p<0.001****p<0.0001.

Finally, cytokine production from CD3+ CD4-CD8- T cells was analysed (Figure 3.5A-D). No significant differences in the proportion of cells expressing IFNγ, IL-22 or GM-CSF in CD4-CD8- T cells between PBMC and SFMC from ERA JIA patients was observed (Figures 3.5E, 3.5G and 3.5H). In contrast, significantly greater number of IL-17+ CD4-CD8- T cells were observed in the SFMC of ERA JIA patients compared to their PBMC (ERA JIA PBMC median= 0.43 IQR=0.22-1.01, ERA JIA SFMC median= 1.05 IQR=0.56-1.65)(p=0.014; Figure 3.5F). In addition, CD4-CD8- T cells from PBMC and SFMC of ERA JIA patients had increased IL-17 production compared to similar cells from oligo-JIA patients from the same compartments (ERA JIA PBMC median= 0.43 IQR=0.22-1.01; oligo JIA PBMC median= 0.14 IQR=0.12-0.15; ERA JIA SFMC median= 1.05 IQR=0.56-1.65; oligo JIA SFMC median= 0.15 IQR=0.12-0.43) (PBMC p=0.028 and SFMC=0.001) (Figure 3.5F). Interestingly more CD4-CD8- T cells from the PBMC from ERA JIA patients produced IFNγ and IL-22 compared to PBMC from oligo JIA patients (for IFNγ: ERA JIA PBMC median= 38.10 IQR=25.68-48.23; oligo JIA PBMC median= 4.90 IQR=4.19-7.60; for IL-22: ERA JIA PBMC median= 0.33 IQR=0.26-0.54; oligo JIA PBMC median= 0.0 IQR=0.0-0.04) (p=0.0044 and p=0.0055 respectively; Figures 3.5E and 3.5G). There was also an enrichment of IL-22 + CD4-CD8- T cells in the SFMC of ERA JIA patients compared to oligo-JIA SFMC (ERA JIA SFMC median= 0.36 IQR=0.26-0.73; oligo JIA SFMC median= 0.19 IQR=0.03-0.39) (p=0.047) (Figure 3.5G).


ERA
aHC
cHC
PBMC
SFMC
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IFNγ
62.1
21.2
40.1
30.1
B
IL-17
0.16
0.18
0.21
0.71
C
IL-22
0.19
0.13
0.24
0.45
D
GMCSF
1.37
N/A
3.81
6.49
CD3
E
IFNγ+ as % of total CD4-CD8- T cells
F
IL-17+ as % of total CD4-CD8- T cells
G
IL-22+ as % of total CD4-CD8- T cells
H
GMCSF+ as % of total CD4-CD8- T cells
aHC cHC ERA Oligo Poly PsA ERA Oligo Poly PsA
PBMC
SFMC
aHC PBMC SFMC
ERA

Figure 3.5 IL-17-producing CD4-CD8- T cells are enriched in the SFMC of ERA JIA patients.

Cytokine production by CD4-CD8- T cells (CD3+) was analysed by flow cytometry following 4 hours stimulation with PMA and Ionomycin in the presence of Brefeldin A, within PBMC form aHC and cHC (n=12, n=3 respectively) and PBMC and SFMC from ERA-JIA (n=13, n=18), oligo-JIA (n=3, n=8) poly-JIA (n=3, n=4) and PsA-JIA patients (n=3, n=3). (A-D) Representative plots showing the gating strategy for identification of cytokine positive CD4-CD8- T cells from aHC, cHC and PBMC and SFMC from an ERA JIA patient for (A) IFNγ; (B) IL-17A; (C) IL-22 and (D) GM-CSF. (E-H) Summary plots of cytokine positive CD4 T cells for (E) IFNγ; (F) IL-17A; (G) IL-22 and (H) GM-CSF. GM-CSF was analysed only in the ERA group, n=6 for both PBMC ad SFMC. Bars represent median values. Statistical analysis by Kruskal Wallis and Mann-Whitney. *p<0.05 **p<0.01 ***p<0.001 ****p<0.0001.

Overall IL-17+ cells were enriched in the SFMC compared to PBMC of ERA JIA patients in all T cell compartments analysed (Figures 3.3F, 3.4F and 3.5F). These data were next explored for relationships between the populations of T cells expressing IL-17 using Spearman's correlation to account for the non-Gaussian distribution of the data. Strong positive correlations were seen between IL-17+ cells from within the SFMC from ERA JIA patients, analysed as follows:

(a) CD4 T cells analysed against CD8 T cells (n=18, r=0.57, p=0.011) (Figure 3.6A) from within the SFMC of ERA JIA patients;
(b) CD4 T cells analysed against CD4-CD8- T cells (n=18, r=0.49, p=0.038) (Figure 3.6B) from within the SFMC of ERA JIA patients; and
(c) CD8 T cells analysed against CD4-CD8- T cells (n=18, r=0.59, p=0.01) (Figure 3.6C) from within the SFMC of ERA JIA patients.

No significant correlations were seen when IL-17+ populations in the PBMC and SFMC from paired ERA JIA samples were analysed as follows:

(d) CD4 T cells from within the PBMC analysed against CD4 T cells from within the SFMC from ERA JIA patients (n=10, r=0.59, p=0.08) (Figure 3.6D);
(e) CD8 T cells from within the PBMC analysed against CD8 T cells from within the SFMC from ERA JIA patients (n=10, r=-0.15, p=0.65) (Figure 3.6E);

(f) CD4-CD8- T cells from within the PBMC analysed against CD4 T cells from within the SFMC from ERA JIA patients (n=10, r=-0.12, p=0.73) (Figure 3.6F);

Together, these data suggest that the IL-17 signature is strong across different T cell populations in a proportion but not all ERA JIA patients, which is restricted to the inflamed site. This raises the question of whether this synovial IL-17 signature extends beyond the T cell compartment.

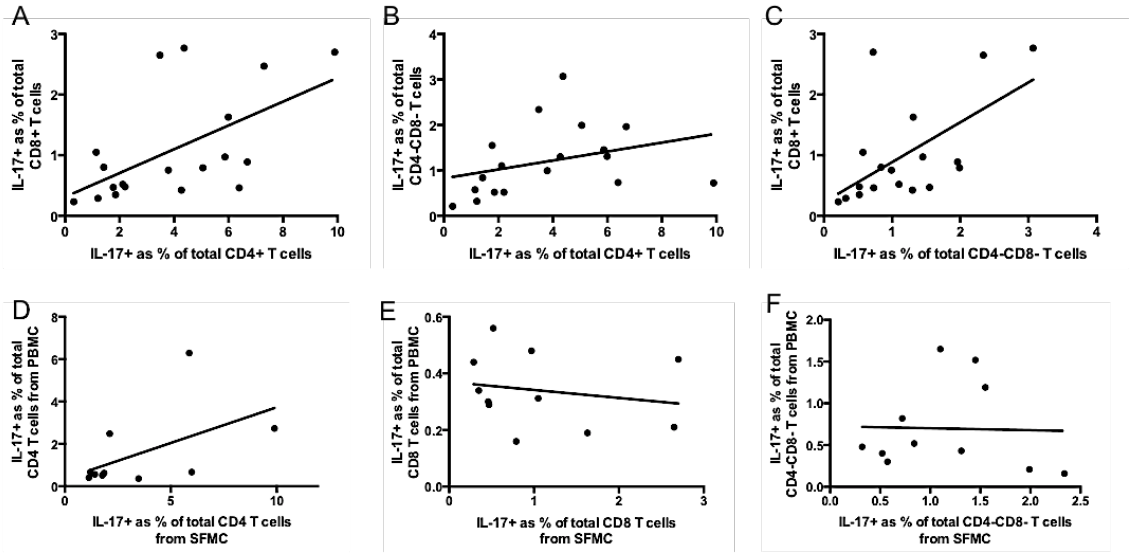


Figure 3.6 ERA JIA patients have a strong IL-17 signature across all T cell subpopulations at the inflamed site.

(A-C) Analysis of IL-17+ T cell subpopulations in the SFMC of ERA JIA patients (n=18) (A) IL-17+CD4 T cells analysed against IL-17+CD8 T cells (r=0.57, p=0.011); (B) IL-17+CD4 T cells analysed against IL-17+CD4-CD8- T cells (r=0.49, p=0.038) and (C) IL-17+CD8 T cells analysed against IL-17+CD4-CD8- T cells (r=0.59, p=0.01). (D-F) Analysis of IL-17+ T cell subpopulations in the PBMC and SFMC from paired ERA JIA patients (n=10). (D) IL-17+CD4 T cells from the PBMC analysed against IL-17+CD4 T cells from the SFMC (r=0.59, p=0.08); (E) IL-17+CD8 T cells from the PBMC analysed against IL-17+CD8 T cells from the SFMC (r=-0.15, p=0.65) and (F) IL-17+CD4-CD8- T cells from the PBMC analysed against IL-17+CD4-CD8- T cells from the SFMC (r=-0.12, p=0.73). Correlation analysis by Spearman correlation.

3.2.3 Presence of dual cytokine positive T cells in ERA JIA.

Polyfunctional T cells, meaning those that co-produce multiple inflammatory cytokines, have been shown to be enriched at the site of inflammation as well as in peripheral blood in oligo-JIA and other types of inflammatory arthritis, are thought of as highly pathogenic, and are associated with more severe disease (Ye et al., 2001, Spreafico et al., 2016). To study the presence of poly-functional IL-17+ T cells in ERA JIA, production of IFNγ and IL-22 from IL-17+ T cell subpopulations was analysed in PBMC and SFMC samples from ERA JIA (n=11, n=15), other JIA subtypes (oligo-JIA (n=3, n=4), poly-JIA (n=2, n=2) and PsA-JIA (n=3, n=3)) and PBMC from aHC (n=13) and cHC (n=3). Additionally, co-production of IL-17 and GM-CSF from T cell subpopulations from PBMC from aHC (n=4) and PBMC (n=6) and SFMC (n=6) from ERA JIA patients by flow cytometry following 4 hours stimulation with PMA and Ionomycin in the presence of Brefeldin A was investigated (Figures 3.7-3.9).

Increased proportions of IL-17/IFNγ double positive cells were identified in CD4 ($p<0.0001$) (ERA JIA PBMC median= 18.50 IQR=15.88-29.65; ERA JIA SFMC median= 64.00 IQR=52.50-69.90) and CD8 ($p=0.0086$) (ERA JIA PBMC median= 58.10 IQR=39.75-77.08; ERA JIA SFMC median= 79.17 IQR=63.60-87.00) T cell compartments within SFMC compared to PBMC from ERA JIA patients (Figure 3.7B and 3.7C). It was interesting to note no difference in IL-17/IFNγ double positive in the CD4-CD8- T cells between PBMC and SFMC (Figure 3.7D). When IL-17/IFNγ double positive cells from the SFMC were analysed between JIA subtypes, ERA JIA patients had a higher number of IL-17/IFNγ double positive CD4 ($p=0.0052$) (ERA JIA SFMC median= 64.00 IQR=52.50-69.90; oligo JIA SFMC median= 47.65 IQR=40.48-54.25) and CD4-CD8- ($p=0.0007$) (ERA JIA SFMC median= 56.46 IQR=50.43-83.70; oligo JIA SFMC median= 26.87 IQR=0.0-38.73) T cells compared to oligo-JIA patients (Figure 3.7B and 3.7D). An expanded IL-17/GM-CSF double positive population was only observed in the CD4 T cell compartment ($p=0.03$) (Figure 3.8B). No differences were seen in the CD8 nor the CD4-CD8- T cell subpopulations (Figure 3.8C and 3.8D).

Only a small proportion of CD4, CD8 and CD4-CD8- T cells were double positive for IL-17 and IL-22 (<20%) and no significant differences were seen between PBMC and SFMC or between JIA subtypes. This finding suggests that IL-17 and IL-22 may be produced by discrete T cell subpopulations (Figure 3.9).

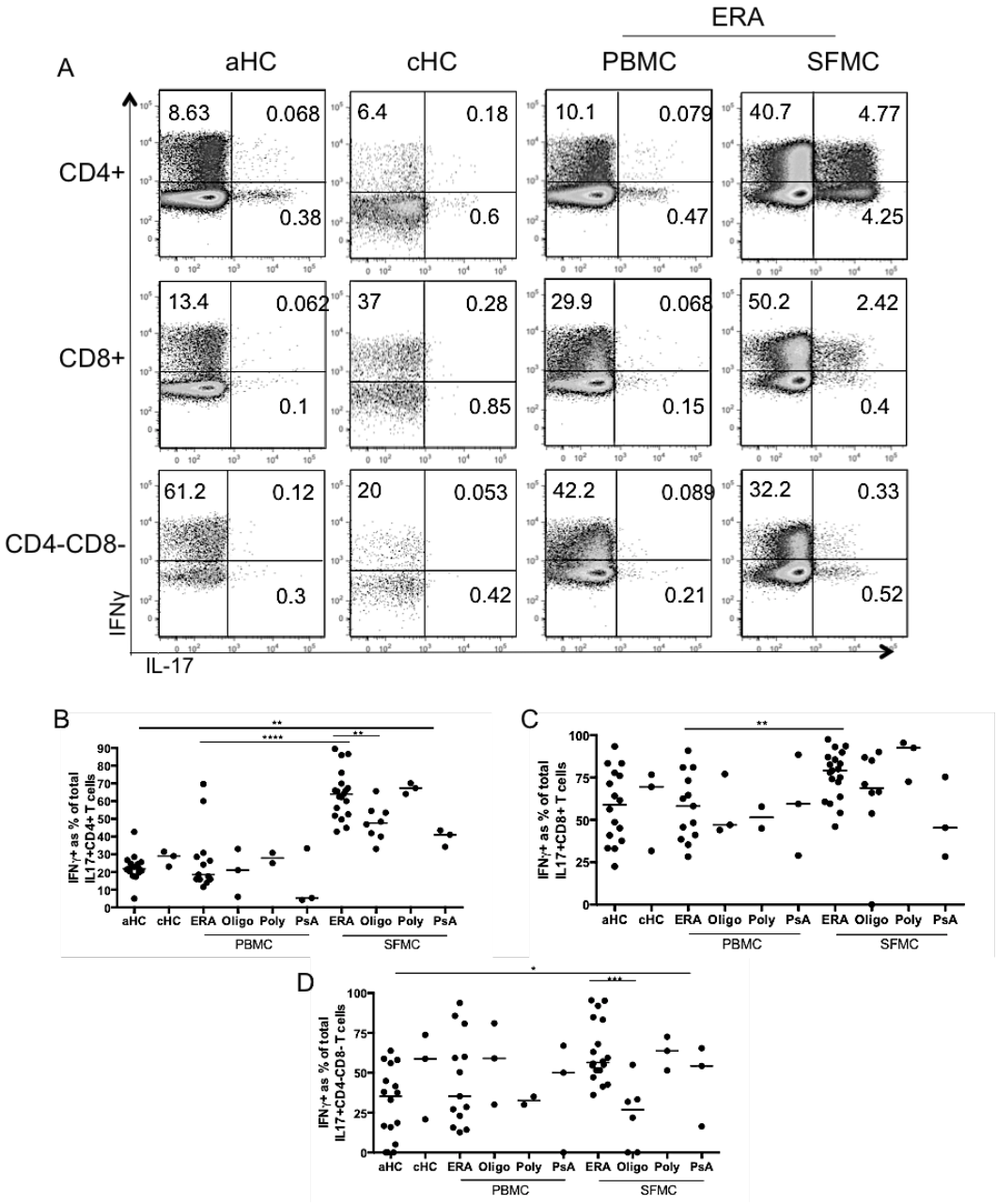


Figure 3.7 IL-17/ IFNγ –double positive T cells are expanded in the SFMC of ERA JIA patients.

Polyfunctional T cells were investigated in PBMC from aHC and cHC (n=12, n=3 respectively) and PBMC and SFMC from ERA-JIA (n=13, n=18), oligo-JIA (n=3, n=8) poly-JIA (n=2, n=3) and PsA-JIA patients (n=3, n=3). (A) Representative flow cytometry plots showing IL-17 and IFNγ staining on CD4 T cells (top row), CD8 T cells (middle row) and CD4-CD8-T cells (bottom row) in PBMC from aHC and cHC and PBMC and SFMC from an ERA JIA patient. (B-D) Summary plots showing IL-17+IFNγ+ cells as a % of total IL-17+ T cells within (B) CD4 T cells; (C) CD8 T cells and (D) CD4-CD8-T cells. Bars represent median values. Statistical analysis by Kruskal Wallis and Mann-Whitney. *p<0.05**p<0.01****p<0.0001.

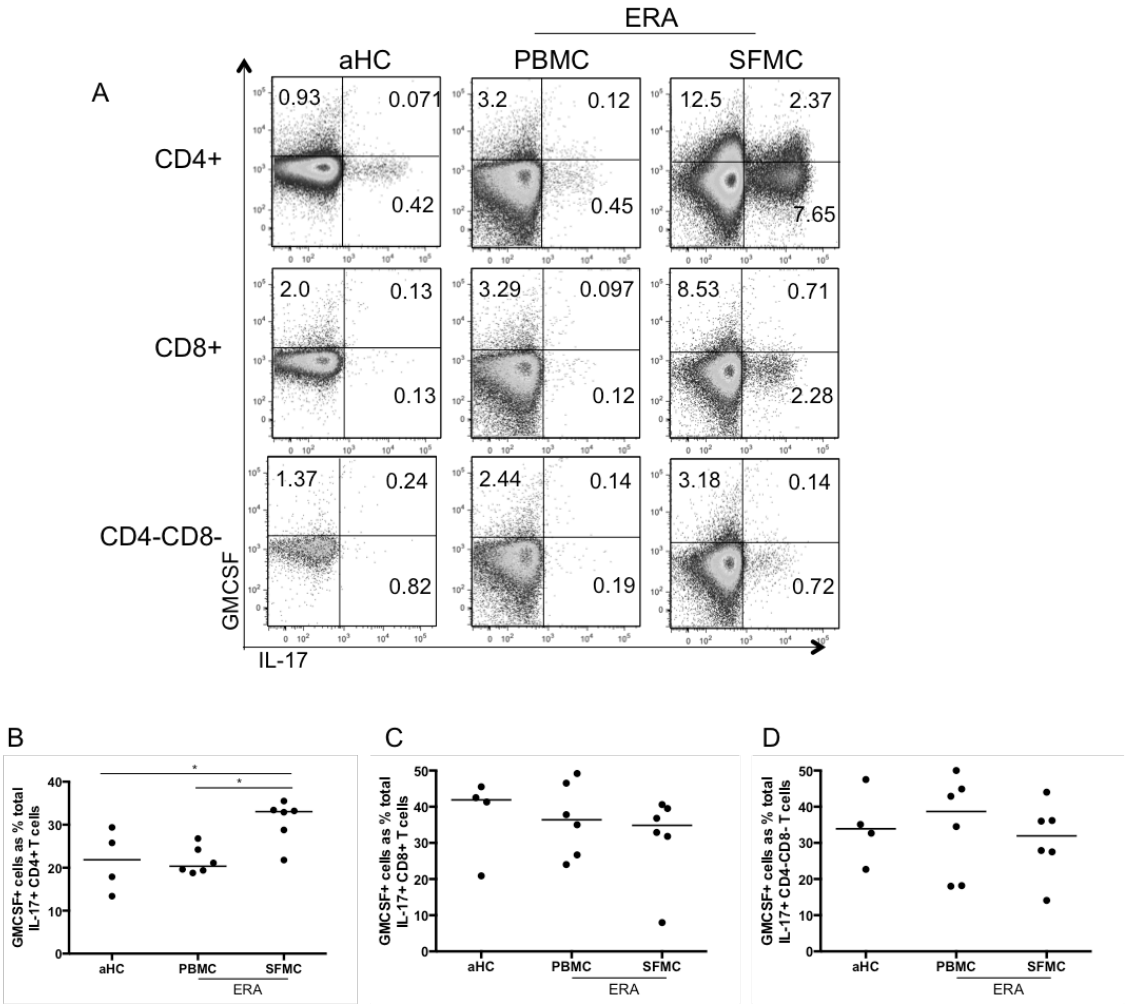


Figure 3.8 IL-17/ GM-CSF–double positive CD4 T cells are expanded in the SFMC of ERA JIA patients.

Polyfunctional T cells were investigated in PBMC from aHC (n=4) and PBMC and SFMC from ERA-JIA (n=6, n=6), (A) Representative flow cytometry plots showing IL-17 and GM-CSF staining on CD4 T cells (top row), CD8 T cells (middle row) and CD4-CD8-T cells (bottom row) in PBMC from aHC and PBMC and SFMC from an ERA JIA patient. (B-D) Summary plots showing IL-17+GM-CSF+ cells as a % of total IL-17+ T cells within (B) CD4 T cells; (C) CD8 T cells and (D) CD4-CD8-T cells. Bars represent median values. Statistical analysis by Kruskal Wallis and Mann-Whitney. *p<0.05

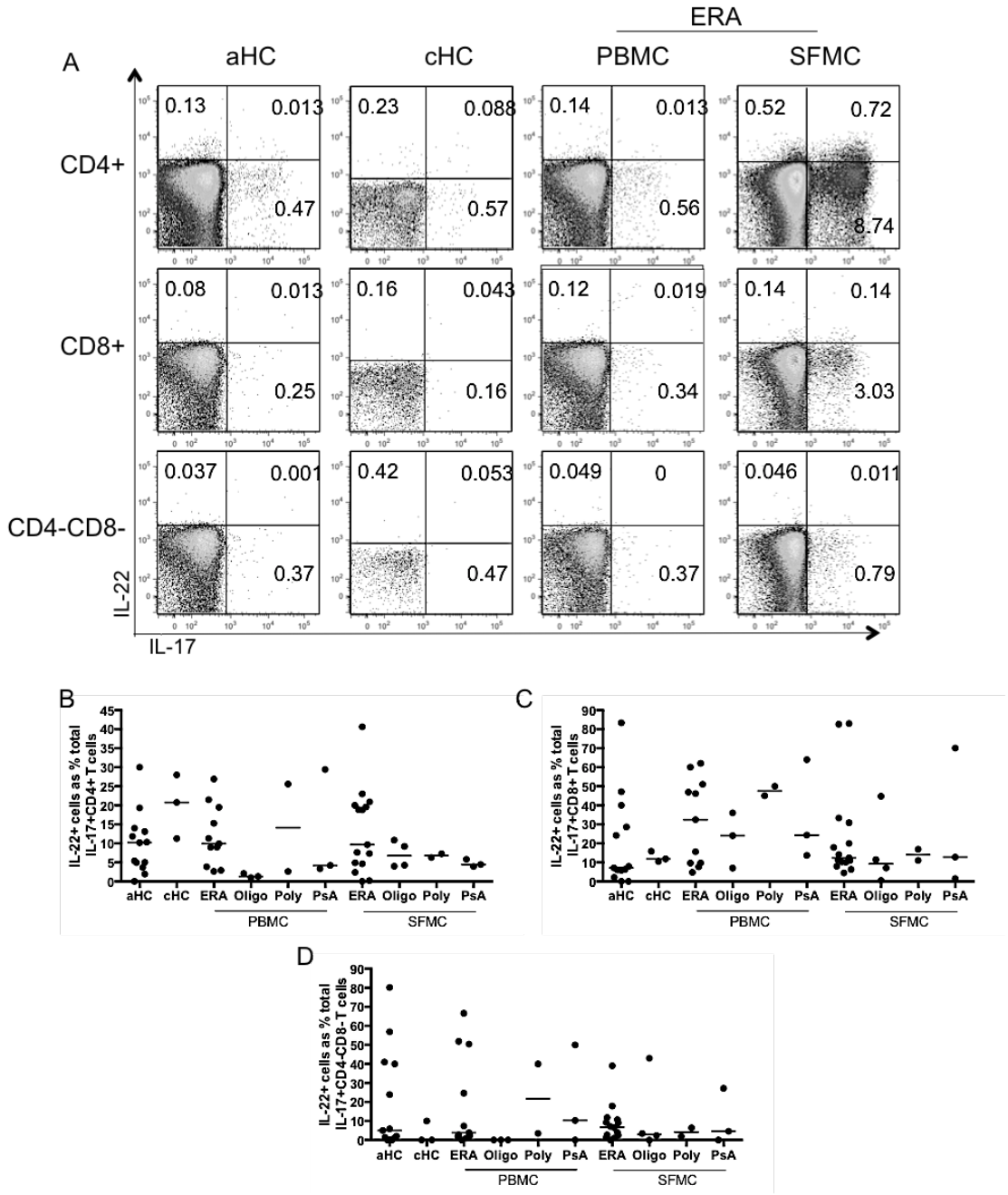


Figure 3.9 IL-17/ IL-22–double positive T cells are not expanded in the SFMC of ERA JIA patients.

Polyfunctional T cells were investigated in PBMC from aHC and cHC (n=12, n=3 respectively) and PBMC and SFMC from ERA-JIA (n=13, n=18), oligo-JIA (n=3,n=8) poly-JIA (n=2,n=3) and PsA-JIA patients (n=3, n=3). (A) Representative flow cytometry plots showing IL-17 and IL-22 staining on CD4 T cells (top row), CD8 T cells (middle row) and CD4-CD8-T cells (bottom row) in PBMC from aHC and cHC and PBMC and SFMC from an ERA JIA patient. (B-D) Summary plots showing IL-17+IL-22+cells as a % of total IL-17+ T cells within (B) CD4 T cells; (C) CD8 T cells and (D) CD4-CD8-T cells. Bars represent median values.

3.2.4 IL-17 positive CD4 T cells are weakly associated with more severe disease.

In order to explore the significance of IL-17+ subpopulations in relation to ERA JIA disease severity, IL-17+ CD4 T cells were analysed as a proportion of total CD4 T cells and correlated to measures of disease severity. Active joint count, ESR and physicians VAS (0.0-10.0 with 0 signifying no active disease, 10 worst disease activity) at time of joint sample were included and correlations were assessed by Spearman's correlation to account for non-Gaussian data. IL-17+ CD4 T cells were weakly associated with the number of active joints ($r=0.35$, $p=0.19$) Physician's VAS ($r=0.43$, $p=0.16$) and at the time of synovial fluid sample collection, although neither of these associations reached statistical significance (Figure 3.10A and 3.10C). No correlation was found between IL-17+ CD4 T cells and the ESR at the time of synovial fluid sample collection ($r=0.16$, $p=0.62$).

Since polyfunctional T cells are generally considered to be more pathogenic, IL-17/IFNγ double positive CD4 T cells were also analysed against active joint count, ESR and physicians VAS (0.0- 10.0) at time of joint sample. Double positive cells were not more closely associated with these measures of disease severity compared to total IL-17+ CD4 T cells. However, they maintained similar associations with active joint count ($r=0.36$, $p=0.19$) and Physician's VAS ($r=0.38$, $p=0.22$) at the time of synovial fluid sample collection (Figure 3.10D and 3.10F). Again, no correlation was found between IL-17/IFNγ double positive CD4 T cells and the ESR at the time of synovial fluid sample collection ($r=0.02$, $p=0.95$). A weak correlation was found when IL-17+ CD4 T cells were analysed against the age of the patient at the time of joint sample, although this did not reach significance ($r=0.41$, $p=0.086$) (Figure 3.11A). Interestingly, IL-17+ CD4 T cells were not preferentially expanded in the SFMC from patients who were positive for the HLA-B27 allele (Figure 3.11B). In this study, treatment with MTX prior to or at time of joint sample did not significantly affect the proportion of IL-17+ CD4 T cells at the inflamed site (Figure 3.11C).

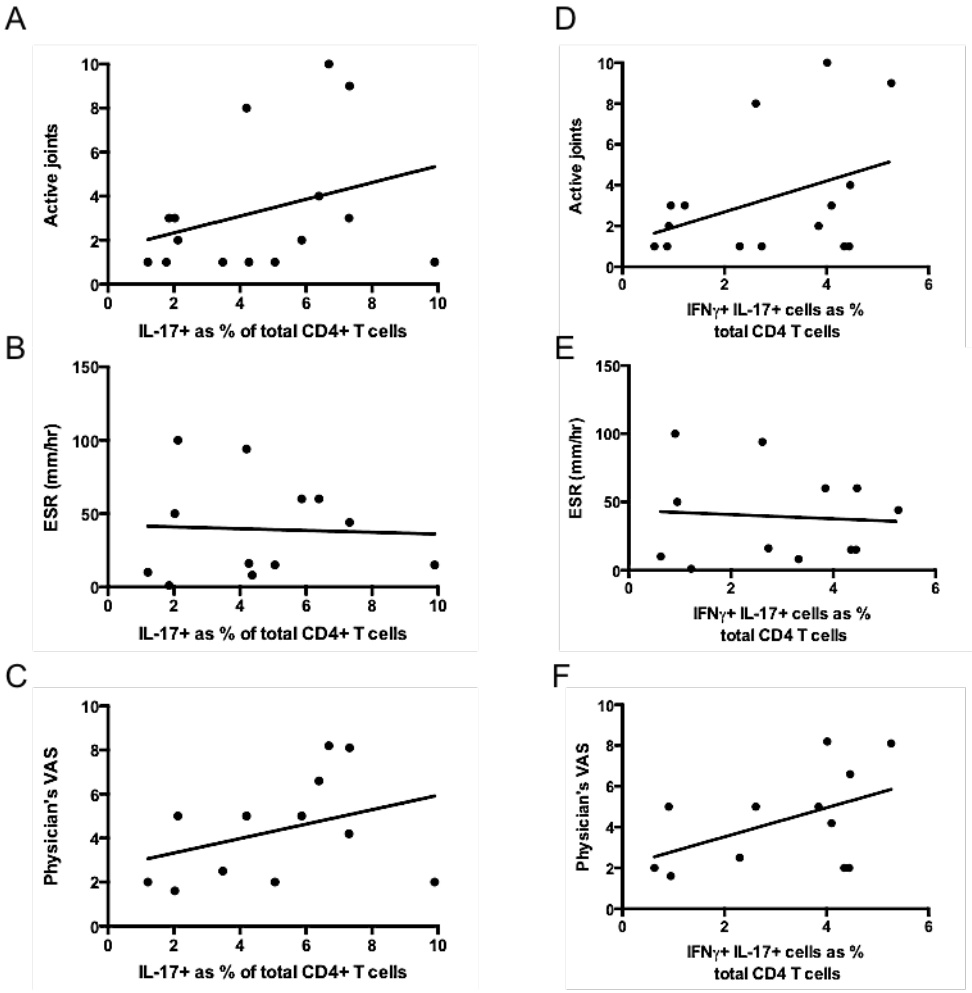


Figure 3.10 Proportion of IL-17-producing CD4 T cells in the SFMC of ERA JIA patients are weakly associated with more severe disease.

(A-C) Correlations in the SFMC of ERA JIA patients between IL-17+ CD4 T cells analysed as a % of total CD4 T cells and clinical measures of disease severity. IL-17+ CD4 T cells analysed against (A) Active joint count at time of sample (n=15, r=0.35, p=0.2); (B) ESR at time of sample (n=12, r=0.16, p=0. 62) and (C) Physician's VAS as time of sample (n=12, r=0.43, p=0.16). (D-F) Correlations in the SFMC of ERA JIA patients between IL-17+IFNγ- double positive CD4 T cells analysed as a % of total CD4 T cells and clinical measures of disease severity. IL-17+IFNγ+ CD4 T cells analysed against (D) Active joints at the time of sample (n=15, r=0.36, p=0.19); (E) ESR at time of sample (n=12, r=0.02, p=0.95); (F) Physicians VAS (n=12, r=0.38, p=0.22). Correlation analysis by Spearman correlation.

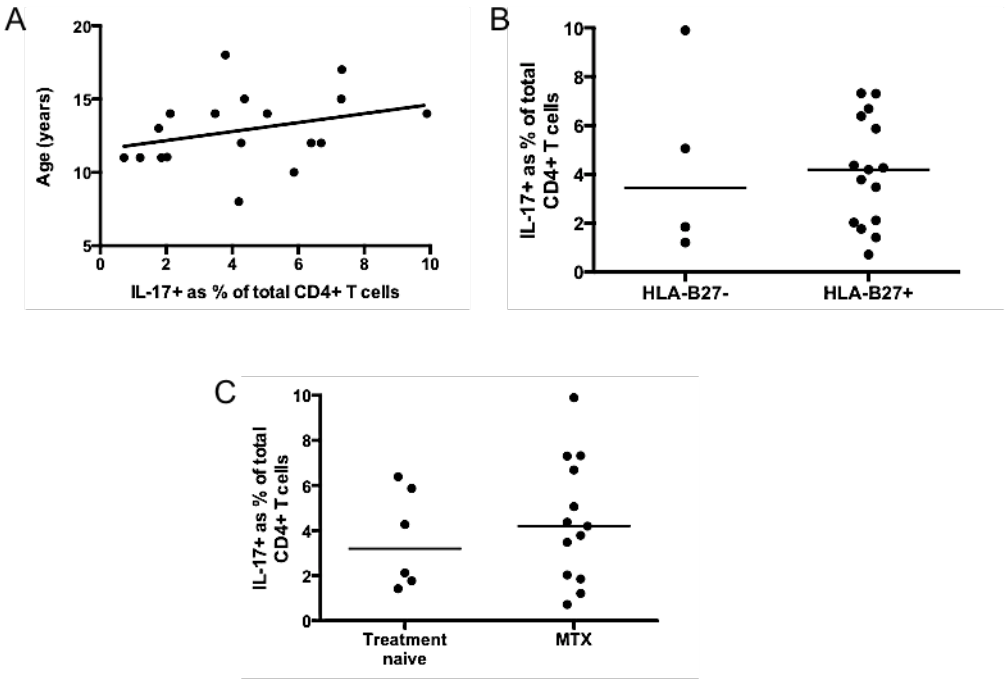


Figure 3.11 Proportion of IL-17-positive CD4 T cells within the synovial fluid T cells is correlated with patient age at time of sampling.

(A) Correlation analysis between IL17+ CD4 T cells and age at time of sample in SFMC of ERA JIA patients (n=18, r=0.41, p=0.086). (B) Summary plot showing IL-17+ CD4 T cells in the SFMC from ERA JIA patients grouped according to carriage of the HLA-B27 allele (HLA-B27- n=4, HLA-B27+ n=14). (C) Summary plot showing IL-17+ CD4 T cells in the SFMC from ERA JIA patients grouped according to treatment received within the last 6 months (treatment naïve n=6, HLA-B27+ n=12). Correlation analysis by Spearman correlation.

3.2.5 'Ex'-IL-17-producing T cells are enriched in the SFMC from ERA JIA patients.

CD161 expression has been linked to expression of *RORC,* the master transcription factor of IL-17 producing T cells, and is associated with T cells with the potential for IL-17 production(Ivanov et al., 2006, Maggi et al., 2010). Therefore, CD161 protein was analysed on CD4, CD8 and CD4-CD8- T cells from aHC PBMC and PBMC and SFMC from ERA JIA patients by flow cytometry, and IL-17/CD161 co-expression was also assessed (gating shown in Figure 3.12A). CD161+ CD4 and CD8 T cells were expanded in the SFMC from ERA JIA patients compared to paired PBMC or aHC PBMC (CD4 T cells: ERA JIA PBMC median= 16.00 IQR=11.18-21.78; ERA JIA SFMC median= 55.90 IQR=41.05-62.30; CD8 T cells: ERA JIA PBMC median= 5.46 IQR=2.98-9.14; ERA JIA SFMC median= 16.40 IQR=11.31-21.03) (Figure 3.12B and 3.12C). Interestingly, fewer CD161+ CD4-CD8- T cells were seen within the SFMC from ERA JIA patients compared to paired or aHC PBMC (ERA JIA PBMC median= 45.25 IQR=24.90-60.90; ERA JIA SFMC median= 35.05 IQR=23.53-44.13) (Figure 3.12D). As expected IL-17+ T cells were also predominantly positive for CD161 expression, although fewer IL-17+CD8 T cells were positive for CD161 (Figures 3.12E-G). No differences in CD161+IL-17+ T cells were seen between PBMC from aHC or ERA JIA patients and SFMC from ERA JIA patients (Figures 3.12E-G). CD161 was expressed on >60% of all IL-17+ CD4 and CD4-CD8-T cells (Figures 3.12E and 3.12G). Typically within CD8+ cells the proportion of IL-17+ cells expressing CD161 was lower than compared to other IL-17+ T cell populations (Figure 3.12 E-G).

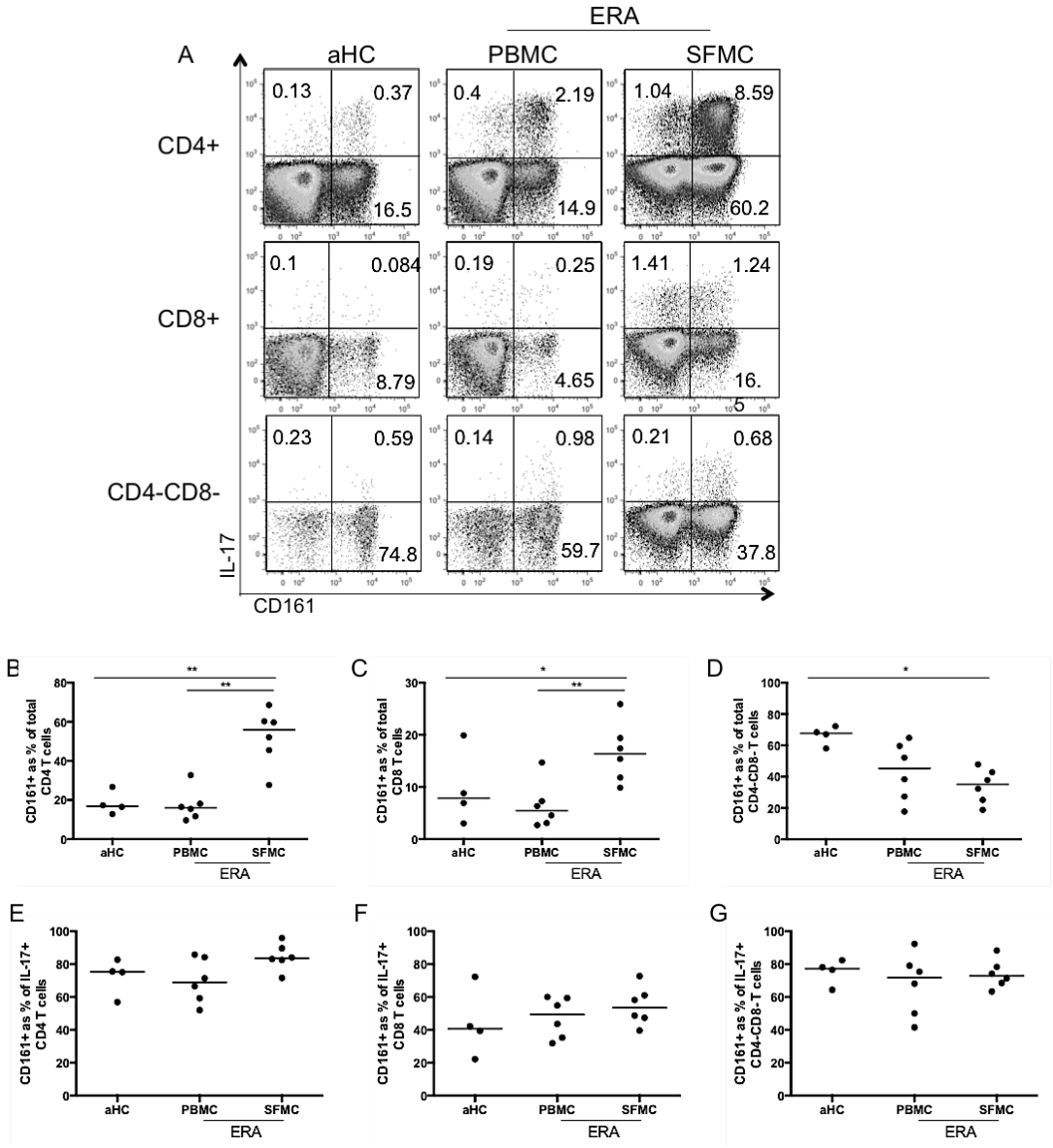


Figure 3.12 T cells expressing CD161 are enriched in the inflamed joints of patients with ERA JIA.

(A) Representative flow cytometry plots showing gating strategy for CD161+ cells, analysed against IL-17, from within the CD4 (top row), CD8 (middle row) and CD4-CD8- (bottom row) T cells, in PBMC from aHC (left column) and PBMC (middle column) and SFMC (right column) of an ERA JIA patient. (B-D) Summary plots for CD161 positive cells as % of T cells populations for (B) CD4 (C) CD8 and (D) CD4-CD8- T cells within the PBMC from aHC (n=4) and PBMC (n=6) and SFMC (n=6) from ERA JIA patients (E-G) Summary plots for CD161 positive cells as % of IL-17A positive T cells (E) CD4 (F) CD8 and (G) CD4-CD8- T cells within the PBMC from aHC (n=4) and PBMC (n=6) and SFMC (n=6) from ERA JIA patients. Statistical analysis by Kruskal Wallis and Mann-Whitney. *p<0.05**p<0.01.

Previous studies from the Wedderburn group, and others have identified CD161 as a marker for a population of IFNγ+IL-17- CD4 T cells with a Th17 ancestry, so called “ex Th17” cells, with pathogenic potential (Nistala et al., 2010a, Maggi et al., 2010, Nistala, 2011, Maggi et al., 2012). This population, as well as its CD8 and CD4-CD8- counterparts were analysed in the PBMC from aHC and the PBMC and SFMC from ERA JIA patients following 4 hour stimulation with PMA and Ionomycin in the presence of Brefeldin A (Figures 3.13, 3.14 and 3.15). IFNγ+IL-17- CD4 T cells were identified as shown in Figure 3.13A and CD161 expression was assessed on this population (Figure 3.13B and 3.13C). CD161 was expressed on 50% of these cells (aHC PBMC median= 60.05 IQR=53.08-64.40; ERA JIA PBMC median= 43.90 IQR=40.53-52.80; ERA JIA SFMC median= 58.30 IQR=51.18-65.65). No significant differences were seen between the proportion of CD161+ cells within IFNγ+IL-17- CD4 T cells between PBMC and SFMC form aHC or ERA JIA patients (Figure 3.13C). CD161+ and CD161- IFNγ+IL-17- CD4 T cells were subsequently analysed for production of the IL-17-associated cytokines, IL-22 and GM-SCF (Figure 3.13D). There was a significant enrichment of GM-CSF+ cells within the IFNγ+IL-17- CD4 T cell population in the SFMC of ERA JIA patients compared to paired and aHC PBMC ($p>0.0001$; Figure 3.13D). Additionally, there was a significant expansion of GM-CSF + cells within the IFNγ+IL-17- CD4 T cell population in the CD161+ compared to the CD161-population ($p=0.011$) (Figure 3.13E). IL-22+ cells within the IFNγ+IL-17- CD4 T cell population were not significantly different between PBMC and SFMC, or between CD161- and CD161+ populations (Figure 3.13F). Similarly, GMCSF/IL-22 double positive cells were not significantly different between PBMC and SFMC, or between CD161- and CD161+ populations (Figure 3.13G).

The same analysis was applied to CD8 T cells (Figure 3.14) and CD4-CD8- T cells (Figure 3.15). Fewer IFNγ+IL-17- CD8 T cells expressed CD161, compared to their CD4 counterparts, where <40% of all cells expressed the marker (aHC PBMC median= 38.60 IQR=23.68-41.75; ERA JIA PBMC median= 17.45 IQR=13.60-32.13; ERA JIA SFMC median= 20.30 IQR=15.33-24.33). Again, no differences in the proportion of CD161+ cells were seen within the

IFNγ+IL-17- CD8 T cell population between PBMC and SFMC. As was seen in CD4 T cells, GM-CSF-positive cells within the IFNγ+IL-17- CD8 T cell population were expanded in the SFMC ($p<0.05$) and were significantly enriched in the CD161 population ($p=0.015$)(Figure 3.14E). Neither IL-22+ cells, nor GM-CSF/IL-22 double positive cells within the IFNγ+IL-17- CD8 T cell population were significantly different between PBMC and SFMC, or between CD161- and CD161+ populations (Figures 3.14F and 3.14G).

A high proportion of IFNγ+IL-17- CD4-CD8- T cells from aHC and ERA JIA PBMC expressed CD161 (>60%) compared to IFNγ+IL-17- CD4-CD8- T cells from within the SFMC from ERA JIA patients (aHC PBMC median= 78.20 IQR=74.48-86.65; ERA JIA PBMC median= 64.55 IQR=53.50-92.90; ERA JIA SFMC median= 47.10 IQR=39.30-66.75) (Figure 3.15C). Again, GM-CSF+ cells were enriched within the IFNγ+IL-17- CD4-CD8- T cell population in the SFMC compared to patient or aHC PBMC ($p<0.01$), but no differences were seen between CD161+ and CD161- populations (Figure 3.15E). Finally, neither IL-22+ cells, nor GM-CSF/IL-22 double positive cells were significantly different within the IFNγ+IL-17- CD4-CD8- T cell population between PBMC and SFMC, or between CD161- and CD161+ populations (Figures 3.15F and 3.15G).

Together these results show that synovial CD161+ cells may share functional similarities with IL-17+ T cells, including increased GM-CSF production.

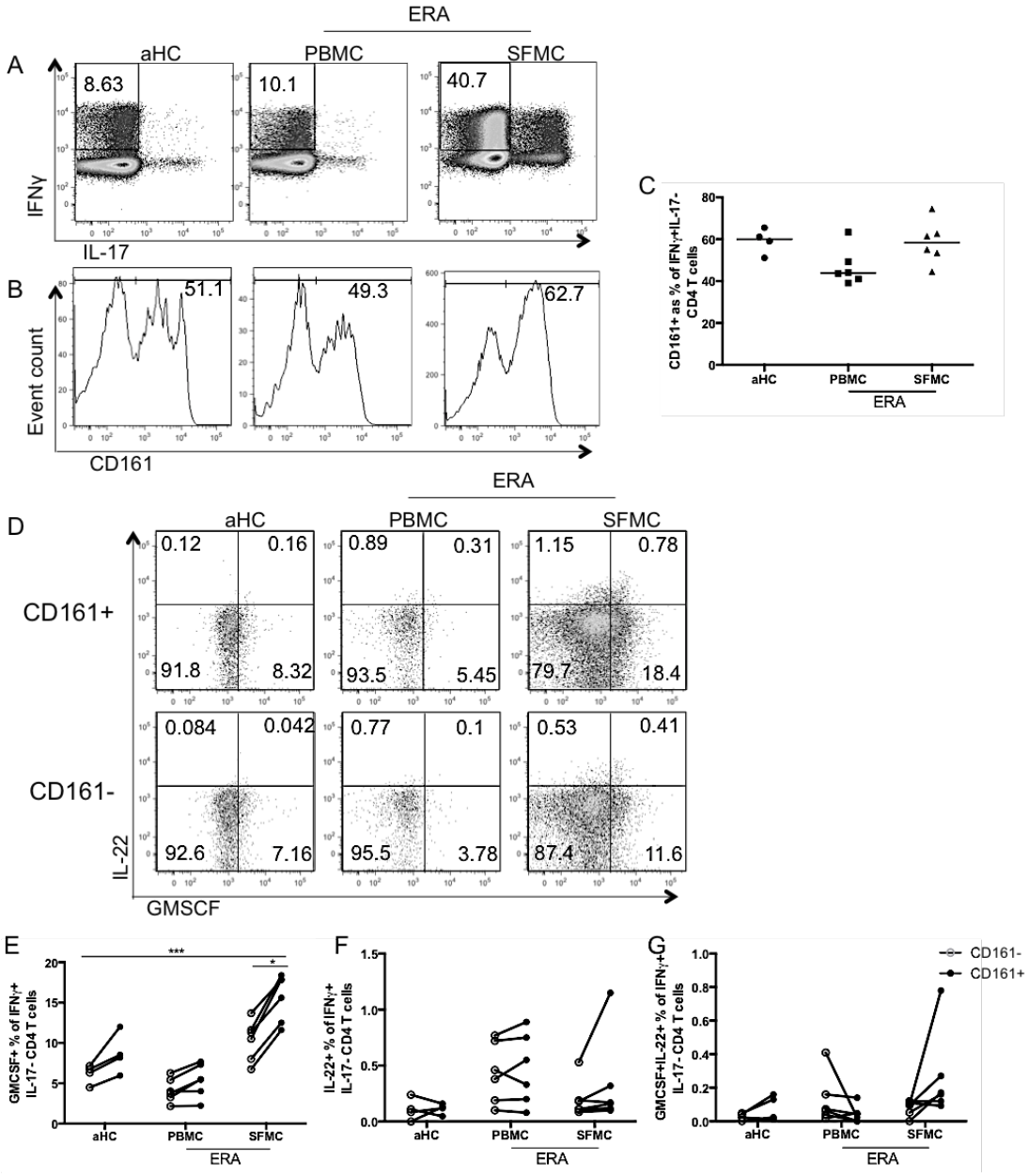


Figure 3.13 Polyfunctional CD4 T cells express CD161 and are expanded at the inflamed site.

(A-C) IFNγ+IL-17- T cells were analyses for expression of CD161 in PBMC from aHC (n=4) and PBMC and SFMC from ERA JIA patients (n=6)(A) Representative gating strategy for the identification of IFNγ+IL17- CD4 T cells. (B) Representative histogram showing the identification of CD161+ and CD161- cells. (C) Summary plot showing CD161+ cells as a proportion of IFNγ+IL-17-CD4 T cells. (D-G) CD161+ and CD161- cells were analysed for coproduction of GM-CSF and IL-22. (D) Representative flow cytometry plots showing GM-CSF and IL-22 staining on IFNγ+IL-17-CD4 T cells within the CD161+(top row) and CD161- populations (bottom row) in PBMC from aHC and PBMC and SFMC from an ERA JIA patient. (E-G) Summary plots comparing polyfunctionality between CD161+ and CD161- IFNγ+IL-17-CD4 T cells analysing cells positive for (E) GM-SCF; (F) IL-22 and (G) GM-CSF and IL-22. Bars represent median values. Statistical analysis by 2-way ANOVA and paired T test. *p<0.05***p<0.001

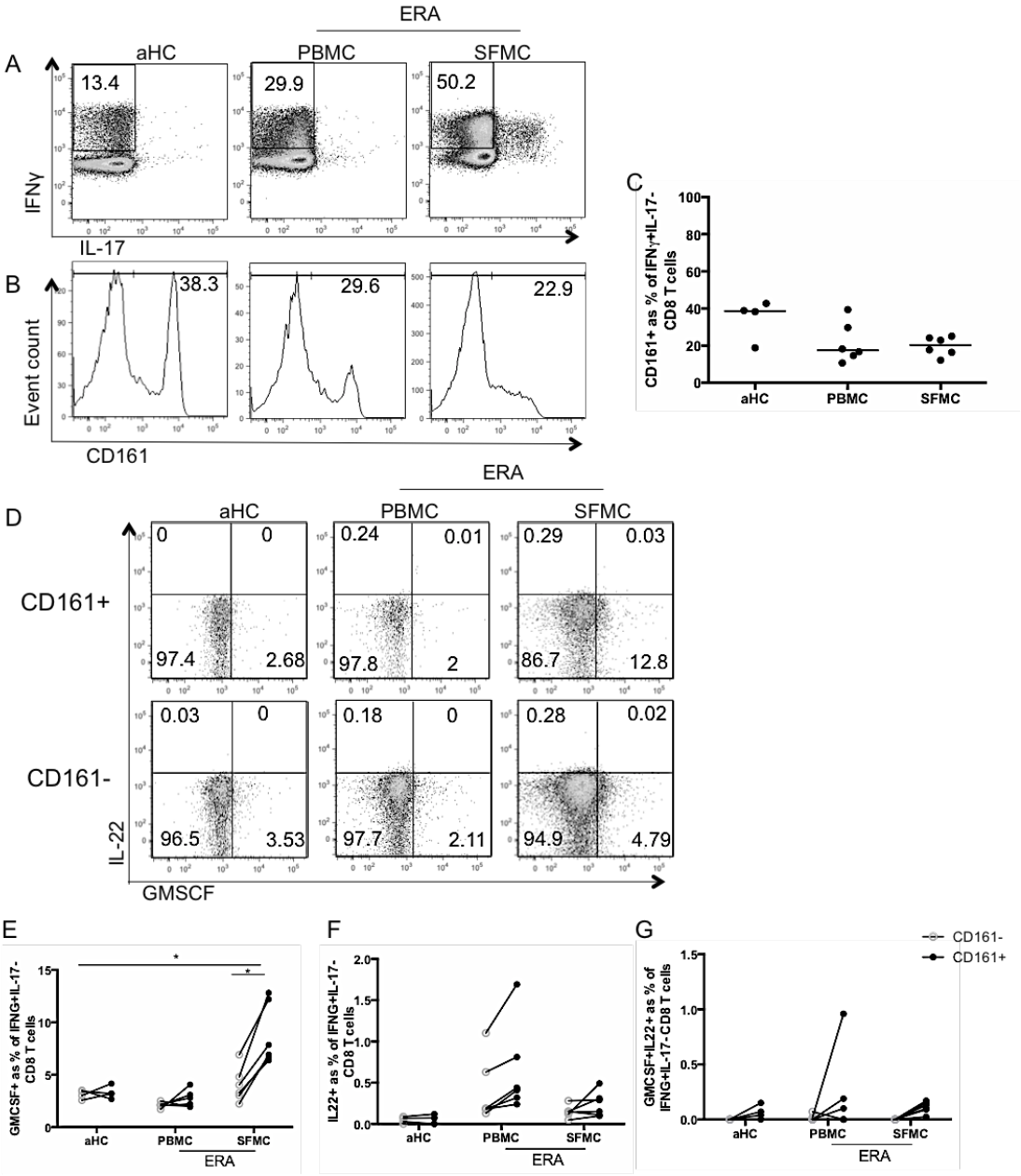


Figure 3.14 Polyfunctional CD8 T cells express CD161 and are enriched at the inflamed site.

(A-C) IFNγ+IL-17- T cells were analysed for expression of CD161 in PBMC from aHC (n=4) and PBMC and SFMC from ERA JIA patients (n=6)(A) Representative gating strategy for the identification of IFNγ+IL17- CD8 T cells. (B) Representative histogram showing the identification of CD161+ and CD161- cells. (C) Summary plot showing CD161+ cells as a proportion of IFNγ+IL-17-CD8 T cells. (D-G) CD161+ and CD161- cells were analysed for coproduction of GM-CSF and IL-22. (D) Representative flow cytometry plots showing GM-CSF and IL-22 staining on IFNγ+IL-17-CD8 T cells within the CD161+(top row) and CD161- populations (bottom row) in PBMC from aHC and PBMC and SFMC from an ERA JIA patient. (E-G) Summary plots comparing polyfunctionality between CD161+ and CD161- IFNγ+IL-17-CD8 T cells analysing cells positive for (E) GM-SCF; (F) IL-22 and (G) GM-CSF and IL-22. Bars represent median values. Statistical analysis by 2-way ANOVA and paired T test. *p<0.05***p<0.001

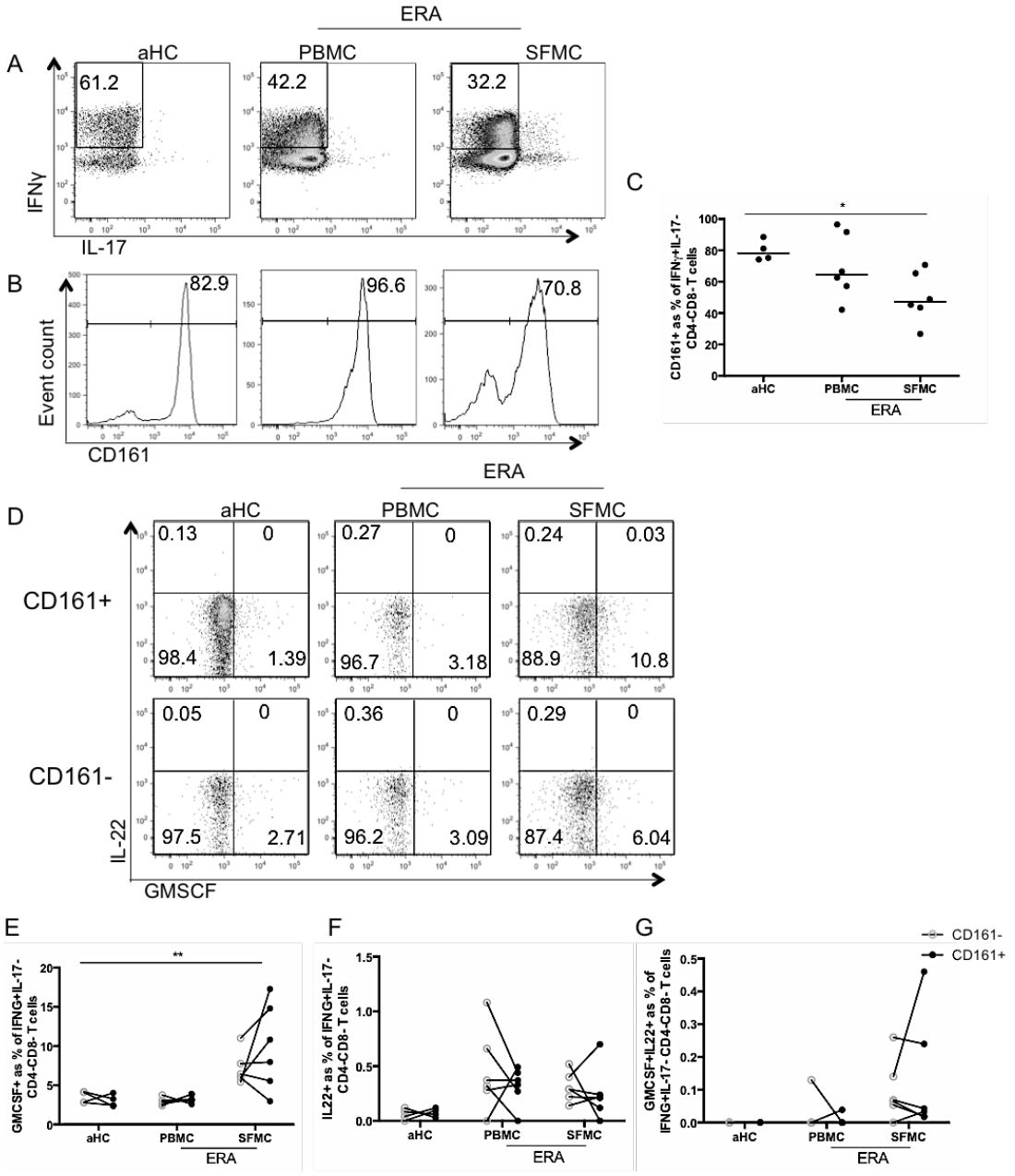


Figure 3.15 Polyfunctional CD4-CD8- T cells are enriched at the inflamed site.

(A-C) IFNγ+IL-17- T cells were analyses for expression of CD161 in PBMC from aHC (n=4) and PBMC and SFMC from ERA JIA patients (n=6)(A) representative gating strategy for the identification of IFNγ+IL17- CD4-CD8- T cells. (B) Representative histogram showing the identification of CD161+ and CD161- cells. (C) Summary plot showing CD161+ cells as a proportion of IFNγ+IL-17-CD4-CD8- T cells. (D-G) CD161+ and CD161- cells were analysed for coproduction of GM-CSF and IL-22. (D) Representative flow cytometry plots showing GM-CSF and IL-22 staining on IFNγ+IL-17-CD4-CD8- T cells within the CD161+(top row) and CD161- populations (bottom row) in PBMC from aHC and PBMC and SFMC from an ERA JIA patient. (E-G) Summary plots comparing polyfunctionality between CD161+ and CD161- IFNγ+IL-17-CD4-CD8- T cells analysing cells positive for (E) GMSCF; (F) IL-22 and (G) GM-CSF and IL-22. Bars represent median values. Statistical analysis by 2-way ANOVA and paired T test. *p<0.05***p<0.001

3.2.6 ERA JIA patients have an expanded memory CD4 population compared to healthy controls

It is known that the memory T cell compartment is significantly expanded in the SFMC from JIA patients (Silverman et al., 1993, Wedderburn et al., 2000a, Chiesa et al., 2004)). Additionally, it has been shown that IL-17-producing T cells originate from a memory population in adult AS (Shen et al., 2009). Since IL-17-producing T cells were enriched in the PBMC from some ERA JIA patients, I hypothesized that this expansion may be indicative of an expanded memory T cell population in this compartment. In order to test this hypothesis, naïve (CD45RA+CD45RO-) and memory (CD45RA-CD45RO+) CD4 T cells were enumerated in the PBMC from aHC (n=4) and cHC (n-=3) and the PBMC (n=5) and SFMC (n=6) from ERA JIA patients (Figure 3.16 A and 3.16B). The majority of all CD4 T cells at the inflamed site (median= 90.20 IQR=89.15-95.08) had a memory phenotype (CD45RA-CD45RO+), which was a significant enrichment compared to memory CD4 T cells found within PBMC (median=29.00 IQR=27.30-44.90) from the same patients (p=0.0043). Intriguingly, a larger memory CD4 population was found in the PBMC from ERA JIA patients (median=29.00 IQR=27.30-44.90), compared to cHC PBMC (median=15.00 IQR=8.12-15.30) (p=0.357) (Figure 3.16B).

To further probe the role of memory in the IL-17 signature of ERA JIA patients, cells were stimulated for 4 hour with PMA and Ionomycin in the presence of Brefeldin A, and subsequently analysed for IL-17 production after gating on the memory population. Within the memory compartment (CD45RO+), IL-17+ cells were expanded within the PBMC and SFMC of ERA JIA patients, but not PBMC from aHC. (Figures 3.16C and 3.16D). These data suggest that inflammation may be more ‘systemic’ in a proportion of ERA JIA patients, which could account for the expansion of cytokine producing T cells in the PBMC of these patients.

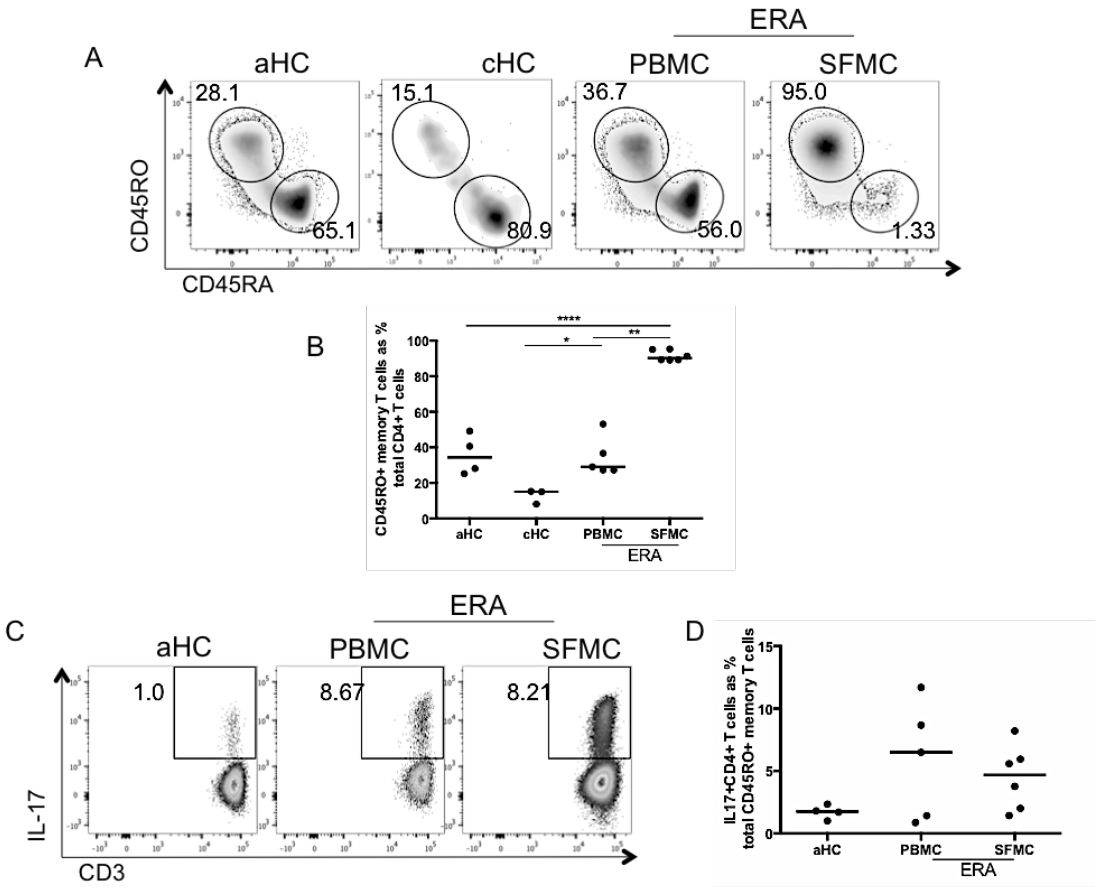


Figure 3.16 Synovial CD4+T cells of ERA JIA patients are predominantly of the memory phenotype.

(A) Representative flow cytometry plots showing gating of naïve (CD45RA+CD45RO-) and memory (CD45RA-CD45RO+) CD4 T cells from PBMC from aHC and cHC and PBMC and SFMC from ERA JIA patients. (B) Summary plot showing CD45RA-CD45RO+ memory cells as a % of total CD4 T cells from PBMC from aHC and cHC (n=4 and n=3 respectively) and PBMC and SFMC from ERA JIA patients (n=5, n=6). PBMC from aHC and PBMC and SFMC from ERA JIA patients were stimulated for 4 hours with PMA and Ionomycin in the presence of BrefeldinA and the naïve/ memory status of the IL-17+ CD4 T cells was analysed by flow cytometry (C) Representative flow cytometry plots showing gating of IL-17 + cells within the memory (CD45RA-CD45RO+) CD4 T cell population from PBMC from aHC and PBMC and SFMC from ERA JIA patients. (D) Summary plot showing IL-17+ cells as a % of total CD45RA-CD45RO+ memory cells CD4 T cells. Bars represent median values. Statistical analysis by Kruskal Wallis and Mann-Whitney. *p<0.05**p<0.01****p<0.0001.

3.2.7 Treg are inversely correlated with IL-17+CD4 T cells at the inflamed site in ERA JIA

Treg are often expanded at sites of inflammation and have been shown to be enriched in the SFMC from JIA patients (de Kleer et al., 2004, Olivito et al., 2009, Spreafico et al., 2016). Additionally, previous published data from our laboratory has demonstrated an enrichment of cytokine producing Treg, which can be identified by the expression of CD161(Pesenacker et al., 2013). Neither total, nor CD161+Treg have been previously analysed in this cohort of ERA JIA patients. To investigate the contribution of this cell-type, Treg (CD3+CD4+CD127low CD25+FOXP3+) were enumerated in the PBMC of aHC and cHC and the PBMC and SFMC of ERA JIA patients as well as other JIA subtypes (oligo, poly and PsA) (Figure 3.17A).

As has been previously shown, Treg were enriched in the SFMC from JIA patients compared to paired PBMC, and this was observed for all subtypes ($p<0.0001$; Figure 3.17B). However, interestingly the proportion of Treg in SFMC from ERA JIA patients was significantly lower than in SFMC from oligo-JIA ($p=0.0072$), Treg were non-significantly expanded in the SFMC compared to PBMC from ERA JIA patients (Figure 3.17B). In line with previously published data investigating these populations in oligo-JIA (Nistala et al., 2008a), a negative correlation was seen between Treg and IL-17+ CD4 T cells in the SFMC although this association did not reach statistical significance ($r=-0.40$, $p=0.16$) (Figure 3.17C).

CD161+ Treg were also investigated. As hypothesised, a significant enrichment of CD161+ Treg were found in the SFMC compared to PBMC from ERA JIA patients ($p=0.015$). No association was seen between CD161+ Treg and IL-17+ CD4 T cells at the inflamed site ($r=0.051$, $p=0.87$) (Figures 3.17D and 3.17E).

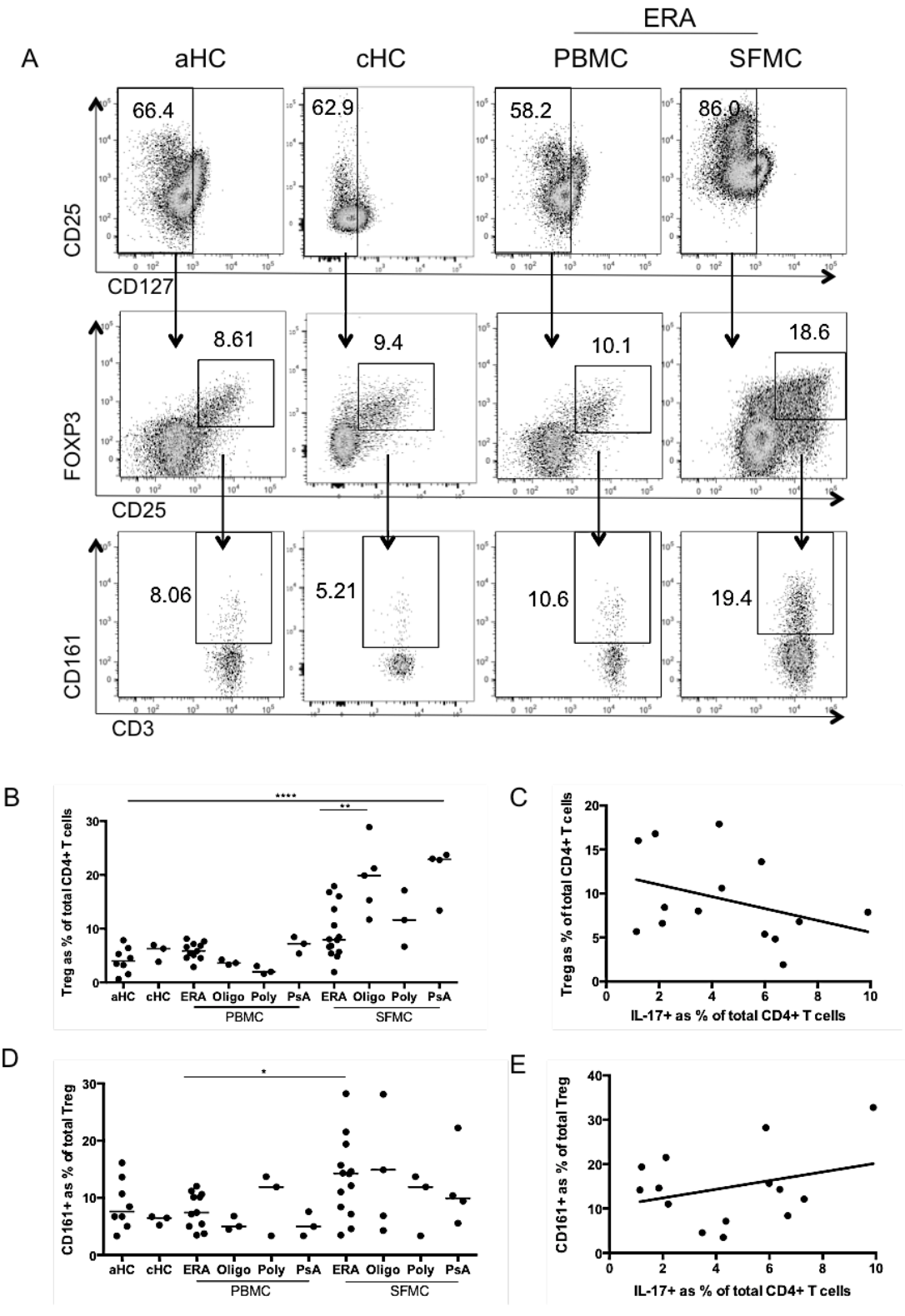

A
aHC
cHC
ERA
PBMC
SFMC
66.4
62.9
58.2
86.0
CD25
CD127
8.61
9.4
10.1
18.6
FOXP3
CD25
8.06
5.21
10.6
19.4
CD161
CD3
B
Treg as % of total CD4+ T cells
aHC cHC ERA Oligo Poly PsA ERA Oligo Poly PsA
PBMC
SFMC
C
Treg as % of total CD4+ T cells
IL-17+ as % of total CD4+ T cells
D
CD161+ as % of total Treg
aHC cHC ERA Oligo Poly PsA ERA Oligo Poly PsA
PBMC
SFMC
E
CD161+ as % of total Treg
IL-17+ as % of total CD4+ T cells

Figure 3.17 Analysis of Treg populations in different JIA subtypes and their relationship to IL-17+ and CD161 expression.

Treg (CD3+CD4+CD127lowCD25+FOXP3+) were enumerated and analysed for expression of CD161 in the PBMC from aHC and cHC (n=8 and n=3 respectively) and the PBMC and SFMC from ERA-JIA (n=11, n=14), oligo-JIA (n=3,n=5) poly-JIA (n=3,n=3) and PsA-JIA patients (n=3, n=4). (A) Representative plots showing the gating strategy for identification of CD161+Treg cells from aHC, cHC and PBMC and SFMC from an ERA JIA patient; (B) Summary plot showing Treg as a % of total CD4 T cells; (C) Correlation analysis showing Treg as a % of total CD4 T cells analysed against IL17+ CD4 T cells as a % of total CD4 T cells in the SFMC of ERA JIA patients (n= 14, r=-0.40, p=0.16); (D) Summary plot showing CD161+Treg as a % of total Treg cells; (E) Correlation analysis showing CD161+ Treg analysed against IL-17+ CD4 T cells in the SFMC of ERA JIA patients (n= 14, r=0.051, p=0.87). Bars represent median values. Statistical analysis by Kruskal Wallis and Mann-Whitney. Correlation analysis by Spearman correlation. *p<0.05**p<0.01***p<0.001****p<0.0001.

3.3 Discussion

The ERA subtype of JIA has a strong genetic association with HLA-B27 and shares many clinical similarities with adult AS (Saurenmann et al., 2007, Layh-Schmitt and Colbert, 2008). While the pathogenesis of AS is associated with the IL-23/IL-17 axis, how closely the pathogenesis of ERA JIA overlaps with AS is not well established. This chapter presents detailed analyses of the immunological phenotype in ERA JIA and confirms an IL-17+ T cell expansion at the inflamed site.

Whilst analysis of SFMC allows unique access to the site of inflammation and provides an insight into the immunopathology, it is not a direct assessment of the cells contained within the synovial tissue at the primary disease site. Due to ethical restrictions, the majority of children undergoing joint fluid aspiration do not have synovial biopsy material sampled. It was therefore not possible to analyse the immune composition within the synovial tissue of JIA patients in this study. The analysis in this thesis is therefore confined to the PBMC and SFMC compartments.

Initial analyses allowed an insight into the cellular composition of the PBMC and SFMC from ERA JIA patients compared to other JIA subtypes as well as healthy control PBMC from aHC and cHC. No significant differences were seen in the relative proportions of the immune cells analysed between JIA subtypes. For the purpose of this chapter only results related to ERA JIA will be discussed, although the same conclusions may also be applied to other JIA subtypes when differences are not specified.

Some noticeable differences were found in the immunological phenotype between PBMC and SFMC in ERA JIA patients. mDC were significantly enriched in the SFMC from ERA JIA patients compared to paired PBMC. This finding builds upon previously published data, which demonstrated an enrichment of mDC subsets in the joints of oligo- and poly-JIA patients (Varsani et al., 2003, Gattorno et al., 2007, Smolewska et al., 2008). During my study, a

population of CD14-CD11c- cells with monocyte morphology was routinely seen, and appeared enriched in the synovial fluid compared to blood of JIA patients. This population may represent activated monocytes that have down-regulated CD14, or a population of lymphoid derived DC (CD11c-CD123+)(Rovati et al., 2008). Although this population was not investigated during this thesis, further examination of this cell population and its possible contribution to pathogenesis would be warranted.

There was a dramatic reduction of B cells noted in the SFMC from ERA JIA patients compared to paired PBMC; a phenomenon that has been previously corroborated in other JIA subtypes (Corcione et al., 2009). Although not investigated in this thesis, B cells in the synovial fluid of JIA patients have been analysed in detail by the Wedderburn group and others (Corcione et al., 2009, Morbach et al., 2011, Botta Gordon-Smith et al., 2015). These studies have shown significant functional differences between blood and synovial B cells including reduced expression of CD73 on synovial B cells, suggesting reduced anti-inflammatory capacity of B cells at the inflamed site (Botta Gordon-Smith et al., 2015). In addition to demonstrating functional diversity in JIA, these studies have also identified associations between synovial B cells and disease severity. The differences observed in synovial B cells act as a reminder that although the relative proportions of immune cells, such as monocytes and NK cells, may not differ significantly between PBMC and SFMC from ERA JIA patients, or may represent only a small proportion of the total mononuclear cell population (B cells), there may be significant functional differences and these cells may remain significant players in disease pathogenesis.

In healthy individuals, a mean value for the ratio of PBMC CD4:CD8 T cells of 2:1 is expected, and, an inverted synovial fluid CD4 to CD8 T cell ratio has been linked to poor prognosis in oligo-JIA (Hunter et al., 2010). An altered CD4 to CD8 ratio (median = 1.02) was demonstrated here within the SFMC from ERA JIA patients, and may be indicative of poor clinical outcomes. Analysis of long-term clinical data from ERA JIA patients could be conducted to elucidate whether this is the case.

Cytokine production was analysed from CD4, CD8 and CD4-CD8- T cell subpopulations within the PBMC of healthy controls and PBMC and SFMC from JIA patients following *ex vivo* stimulation with PMA and Ionomycin in the presence of Brefeldin A. There are some limitations associated with stimulation of this kind, including the supra-physiological strength of the stimulatory signal. Some investigators use an alternative system using stimulation through the TCR, with or without costimulation. Stimulation with PMA and Ionomycin identifies all cells capable of cytokine production at a given time point, rather than a true demonstration of cell function *in vivo*. However, this limitation is mitigated by technical consistency. PBMC and SFMC were stimulated in an identical way throughout this study and therefore variances described between groups are most likely representative of true differences.

For the purpose of cytokine analyses, CD4-CD8- T cells were investigated as a surrogate of the γδ T cell population. Due to insufficient space on the panel, it was not possible to include γδTCR in the cytokine panels, as its inclusion would have resulted in significant technical complications. Although γδ T cells represented the predominant CD4-CD8- population (>80%), analysis of this population should be treated with some caution. It is possible that the cells that did not stain positively for the γδTCR were activated CD4 or CD8 cells that had down regulated their respective markers, or alternatively may represent a small population of NKT cells. The possibility of NKT cell contamination is perhaps supported by the abundance of CD4-CD8-T cells that expressed CD161, particularly within the PBMC from aHC, which was higher than has been previously reported in γδ T cells (Battistini et al., 1997, Poggi et al., 1999). However, in line with the data presented in this Chapter, significant variability of CD161 expression on circulating γδ T cells has been reported, and the high proportion of CD161-expressing cells observed here may not necessarily be indicative of contamination (Kenna et al., 2004). Due to this uncertainty, it is not possible to precisely determine cytokine production by γδ T cells in this study and further analysis with the inclusion of γδTCR should be conducted. However, these data do identify cytokine-producing T cells at the inflamed site of ERA JIA patients and some very cautious conclusions may be drawn.

Th17 cells have been previously implicated in the pathogenesis of JIA as well as adult inflammatory arthropathies (Spreafico et al., 2016). In particular, IL-17 has been heavily implicated in the pathogenesis of adult AS, which shares clinical and genetic features with ERA JIA (Berntson et al., 2008, Shen et al., 2009). This chapter investigated the role of IL-17-producing T cell subsets and their association with disease in ERA JIA patients from a British cohort. A role for IL-17 in pathogenesis of ERA JIA could have dramatic implications for treatment decisions in the future as novel biological therapies targeting the IL-23/IL-17 axis have been developed, and have shown promise in the treatment of adult psoriatic arthritis and AS (Braun et al., 2015).

As hypothesized, and in accordance with similar data collected in an Indian cohort, Th17 cells were enriched in the synovial fluid of ERA JIA patients (Mahendra et al., 2009). In fact, the expansion of IL-17-producing T cells was not restricted to the CD4 compartment in the joints of ERA JIA patients, but extended to CD8 and CD4-CD8- T cells. Although IL-17+ cells were fewer in the CD8 T cell compartment compared to CD4 T cells, the potential contribution of CD8-derived IL-17 should not be underestimated. Here, it has been shown that CD8 T cells are significantly expanded in the joints of ERA JIA patients, and therefore the IL-17+CD8 T cells may be underrepresented by these analyses.

In parallel to IL-17 production, IL-17-associated, GM-CSF+ cells were also expanded in synovial CD4 and CD8 T cell populations from ERA JIA patients. This observation has been previously been reported in oligo-JIA patients (Piper et al., 2014a). Additionally, IL-17+ T cells at the inflamed site showed considerable co-production of inflammatory IFNγ and GM-CSF suggesting a highly pathogenic phenotype. Indeed, polyfunctional T cells have been previously reported in the joints of patients with adult and childhood arthritis, where they are associated with poor clinical outcomes (Aarvak et al., 1999, Piper et al., 2014a, Spreafico et al., 2016). Moreover, analysis of clinical measures of disease severity revealed that Th17 cells at the inflamed site in ERA JIA are weakly associated with more severe disease measured by active joint count and Physician's VAS at the time of sampling, implicating these cells in disease pathogenesis. It was unexpected that double positive, IFNγ/IL-17,

CD4 T cells were not more closely associated with disease severity than single positive Th17 cells. This may be attributed to all cells within the inflamed joint being highly active and therefore more pathogenic than their Th17 counterparts within the PBMC.

The HLA-B27 allele is commonly associated with IL-17 mediated disease, via mechanisms described in Chapter 1. With this in mind, it was perhaps surprising that there was no significant difference found in the proportion of Th17 cells within the SFMC of HLA-B27+ compared to HLA-B27- patients. ~80% of the ERA JIA patients analysed during this study were HLA-B27+. As the number of samples for these analyses from HLA-B27- patients was small, the results are likely to be underpowered and more significant differences may have been seen with larger sample numbers.

Killer cell lectin-like receptor, CD161, is induced by RORC and is associated with IL-17-producing T cells(Cosmi et al., 2008, Maggi et al., 2010). CD161 expression was more common on CD4 and CD8 T cells from the SFMC of ERA JIA patients compared to paired PBMC. This further supports a strong IL-17 signature at the inflamed site in ERA JIA. As expected CD161 was expressed on the majority of IL-17+ CD4 and CD4-CD8- T cell subsets. Distinct populations with low, intermediate and high CD161 expression were seen within IL-17+ CD8 population. Human mucosal associated invariant T (MAIT) cells are defined by very high expression of CD161 and originate from committed IL-17-type CD8 precursors(Walker et al., 2012). These cells have tissue-homing properties and are expanded at sites of inflammation(Billerbeck et al., 2010, Serriari et al., 2014a). It is likely that the $CD161^{high}$ IL-17+CD8 T cells identified represent a population of MAIT cells.

In addition to IL-17+ T cells, an expansion of CD161+IFNγ+ “ex-IL-17” was noted. A previous study from the Wedderburn group has implicated these cells in the pathogenicity of JIA (Nistala et al., 2010a, Nistala, 2011). CD161+IFNγ+IL-17-CD4 T cells expressed higher levels of chemokine receptor CCR6, which has been shown to be crucial for the entry of pathogenic cells to the inflamed sites in rheumatoid arthritis and in animal models (Hirota et al.,

2007). Additionally, these cells are shown to express high levels of IL-23R and produce elevated IFNγ and TNFα in response to IL-23 stimulation (Nistala, 2011). As previously mentioned the HLA-B27 allele is associated with aberrant IL-23 production (DeLay et al., 2009, Goodall et al., 2010). CD161+IFNγ+ T cells are often overlooked in analyses, however, the response of these cells to IL-23 makes this population particularly interesting in the context of ERA JIA and other HLA-B27 associated disease.

Taken together, these cytokine data highlight imprinting of the IL-23/IL-17 axis in ERA JIA pathogenesis. It is noteworthy that IL-17+ cells are expanded in all T cell compartments. The clear expansion of IL-17+ populations at the inflamed site in ERA JIA has significant clinical implications as new biological therapies targeting the IL-23/IL-17 pathway are now gaining momentum for proposed trials in JIA. A simple robust method to detect this synovial IL-17 signature, in the peripheral blood, could be a valuable way to select a subset of patients for these specific targeted therapies.

In addition to IL-17, these analyses also demonstrate an expansion of IL-22+ T cells from all T cell subpopulations (CD4, CD8 and CD4-CD8-). This data corroborates previously published data in adult arthropathies, and may allude to a role for IL-22 in pathogenesis of ERA JIA. Although this hypothesis has not been examined in detail, IL-22 has been implicated in the pathogenesis of other inflammatory conditions including adult rheumatoid arthritis where it has been shown to exacerbate disease by inducing activation and proliferation of synovial fibroblasts(Ikeuchi et al., 2005). Many reports have been made of the close association and co-production of IL-17 and IL-22. There is still no consensus on whether these cytokines arise from a common T cell subset, or whether they represent parallel or plastic populations (Eyerich et al., 2009). Interestingly, there was limited overlap between IL-22 and IL-17 production during this study, suggesting that IL-17 and IL-22 producing cells may be distinct subtypes in this environment.

T cell memory at the inflamed site in ERA JIA has not previously been studied in detail. In line with previous reports of other subtypes, the memory population

represented >90% of all CD4 T cells within the SFMC compared to ~30% seen in paired PBMC (Silverman et al., 1993, Wedderburn et al., 2000a, Chiesa et al., 2004)The memory pool is known to increase with age in response to antigen exposure (Cossarizza et al., 1996, Farber et al., 2014). It is interesting that the memory pool within the PBMC from ERA JIA patients was significantly larger than the memory pool in cHC PBMC (median age = 6.24) and was more aligned with the T cell memory population size in aHC PBMC despite the age difference between these two groups (aHC median = 26.15, ERA JIA PBMC median= 13.22). Additionally, within the gated memory CD4 T cells there was an expansion of IL-17+ cells in the PBMC from ERA JIA patients compared to aHC. This expanded IL-17+ memory population within the PBMC of ERA JIA patients, may be indicative of systemic disease although this is difficult to conclude from the low number of samples analysed. For more accurate analysis it would be prudent to analyse the ERA JIA PBMC memory population against more closely age matched controls and also against PBMC from other JIA subtypes, in a validation cohort.

To investigate regulation by T cells at the inflamed site, FOXP3+Treg populations were analysed. A non-significant enrichment of Treg was seen in the SFMC from ERA JIA patients compared to paired PBMC. However, fewer Treg were identified in the SFMC of ERA JIA patients compared to other JIA subtypes. This is not entirely surprising given the reciprocal relationship of Treg with Th17, shown here and in previous publications, and the dramatic expansion of Th17 in the ERA JIA subtype(Spreafico et al., 2016).

It is likely that these analyses represent an underrepresentation of the true Treg population at the inflamed site en ERA JIA. During the analysis, a population of CD25+FOXP3^{low} cells was observed. This population has also been identified in other JIA subtypes and analysed in detail in the Wedderburn group. These previous studies show that despite reduced expression of the FOXP3 protein, the CD25+FOXP3^{low} population of Treg are demethylated at the Treg ‑ specific demethylated region (TSDR), which is an intronic enhancer region that can be used to discriminate Treg from other FOXP3-expressing cells, where FOXP3

expression may have been upregulated due to T cell activation. Additionally, these FOXP3low cells were shown to maintain their suppressive capacity *in vitro* (Bending et al., 2014, Bending et al., 2015). I therefore believe it would be reasonable to include these cells in future analyses of regulatory T cells in sites of inflammation.

As previously mentioned, CD161 is commonly associated with Th17 cells, but has more recently been identified on a population of FOXP3+Treg with an inflammatory signature (Cosmi et al., 2008, Maggi et al., 2010, Pesenacker et al., 2013). CD161+ Treg were significantly enriched in the joints of ERA JIA patients. CD161+ Treg are demethylated at the TSDR and are suppressive *in vitro.* However, they also have a Th1/Th17 transcriptional signature, are able to produce inflammatory cytokines, including IL-17 and IFNγ, and are enriched in the joints of oligo JIA patients (Pesenacker et al., 2013, Afzali et al., 2013, Duurland et al., 2017b). It is not currently known whether these cells act in an inflammatory or regulatory role in the joints of JIA patients, however cytokine producing Treg have been identified in autoimmune hepatitis where they are implicated in promoting inflammation (Arterbery et al., 2016). CD161+Treg and their inflammatory potential should not be overlooked in ERA JIA, as CD161+Treg may contribute to the IL-17 signature and pathogenesis, and represent another prospective target of biological therapies affecting the IL-23/IL-17 axis.

Taken together, the results presented in this chapter highlight a strong IL-17 signature within the joints of patients with ERA JIA and suggest that similarities between ERA JIA and adult AS may extend beyond clinical features or genetic associations, to key pathological mechanisms in the joint. It is clear that IL-17 'imprinting' is not restricted to the CD4 T effector population, but is also evident in CD8 and γδ T cell populations and has even infiltrated the regulatory T cell compartment. This suggests that synovial milieu is supportive of the recruitment and survival of IL-17 producing cells. I therefore hypothesize that this may extend beyond the T cell compartment, and the ERA JIA synovial environment may also promote IL-17 production from other cell types, which may also contribute to disease pathogenesis. The following chapter will investigate the

innate lymphoid cell (ILC) signature in ERA JIA, with the hypothesis that IL-17+ ILC will also be expanded at the inflamed site.

4 : Defining the Innate Lymphoid Cell Signature in JIA

4.1 Introduction

Innate Lymphoid cells (ILC) are a newly described population of immune cells, which can be divided into three subsets (ILC1, ILC2 and ILC3), which mirror T cell subsets (Th1, Th2 and Th17) cells based on their transcription factor and cytokine profiles. (Spits and Cupedo, 2012).

ILC have been implicated in a plethora of inflammatory and autoimmune diseases including, but not restricted to, asthma(Chang et al., 2011), inflammatory bowel disease(Bernink et al., 2013b), and skin inflammation(Teunissen et al., 2014a). To date, there have been very few reports of ILC involvement in inflammatory rheumatic diseases. Synovial ILC have only been characterised in adult forms of arthritis, in particular psoriatic arthritis, where group 3 ILC were enriched at the inflamed site and were associated with disease severity (Leijten et al., 2015). No studies have been published implicating ILC in JIA.

The aims of this chapter were to characterize the ILC populations at the inflamed site in childhood arthritis and to test the hypotheses that IL-17-producing NCR-ILC3 are enriched in the synovial fluid of JIA, and in particular ERA JIA, patients and are associated with more severe disease. These aims were addressed by:

1. Developing an effective strategy for the identification of ILC populations in synovial fluid from patients with JIA.
2. Enumerating the ILC subpopulations and interrogating functionality of ILC subpopulations at the inflamed site.
3. Probing for associations between ILC populations and clinical measures of disease severity in JIA.

4.2 Results

4.2.1 CD161 is useful for the identification of ILC in synovial fluid

ILC are a relatively newly discovered population of immune cells and until recently there was little consensus on an efficient strategy for their identification. Due to limited published data about ILC and their characteristic cell markers, significant optimisation was required in order to generate a strategy for the identification of ILC in arthritis.

It was first important to consider the most appropriate scatter gate for the identification of ILC. Cells within the SFMC population are typically highly activated and as such have higher forward and side scatter properties than similar cells from within PBMC. During preliminary optimisation assays, ILC were identified as those cells lacking makers associated with known immune cell lineages (B cells, T cells, NK cells, monocytes, DC, stem cells, mast cells and eosinophils), which expressed the IL-7Rα chain (CD127). Antibodies used as listed in Chapter 2. In order to elucidate the best scatter gate, the lineage-CD127+ ILC populations from within the lymphocyte gate (based on forward and side scatter) were compared to the same population from a scatter gate, which included all live cells. No difference was seen between the sizes of the ILC population within the lymphocyte gate compared to the total cell gate in the PBMC of healthy controls (Figure 4.1A). However, a significant difference was seen between the ILC populations from within the two gates in SFMC, with the emergence of a lineage^{low}CD127+ population within events from the total cell gate (Figure 4.1B). Back gating analysis showed that the lineage^{low} populations were larger cells, which appeared to have an intermediate scatter profile, positioned between the lymphocyte and monocyte populations (Figure 4.1C).

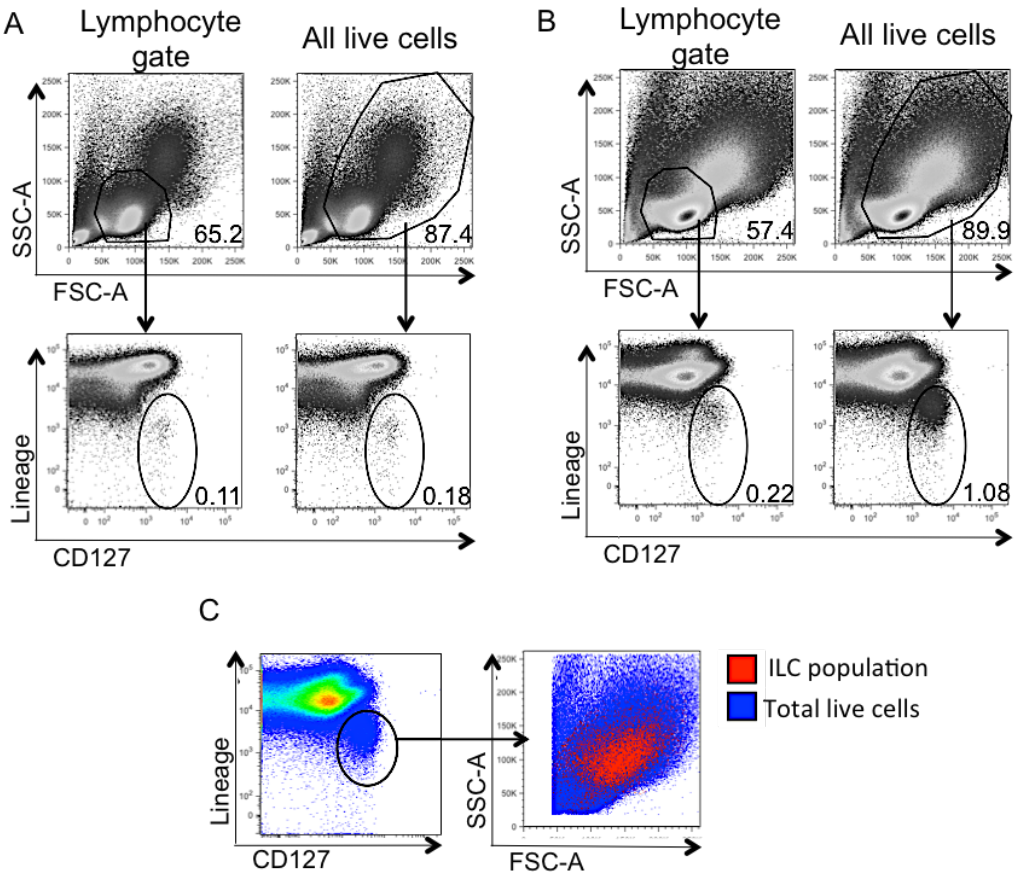


Figure 4.1 Optimisation of gating strategy for the identification of innate lymphoid cells (ILC) in the PBMC and SFMC from JIA patients.

A 'dump' channel (' lineage' as shown) was constructed to identify and exclude cells of known lineages, using antibodies all labelled with the PE fluorochrome against CD1a, CD3, CD11c, CD14, CD16, CD19, CD34, CD94, CD123, BDCA2, FcεRIα, αβTCR and γδTCR. (A, B) Analysis by flow cytometry of (A) PBMC, and (B) SFMC, representative samples. Upper plots show scatter plots gated on the lymphocyte (upper left plots A and B) or total cell gate (upper right plots, A and B) following exclusion of dead cells and doublets (not shown); lower plots show gated events of lineage–CD127+ cell population within the lymphocyte (lower left plots A and B) or total cell gate (lower right plots, A and B); (C) Representative plot of back-gating analysis shows size and granularity of lineage^{low}CD127+ve population from SFMC assessed by forward scatter area (FSC-A) and side scatter area (SSC-A). All gated events form the left hand plot are indicated as red cells on right hand plot.

The NK cell receptor CD161 has been proposed by experts in the field to be an effective marker for the identification of CD127+ ILC subsets in humans(Mjosberg et al., 2011a, Spits et al., 2013). In order to assess the value of CD161 as a tool for the identification of ILC in synovial fluid, healthy control PBMC and SFMC from patients were stained with the ILC flow cytometry panel (containing lineage markers and CD127) with the addition of CD161. CD45 was also included in the amended ILC panel in order to exclude non-leukocytes from analysis (Figure 4.2A).

Results of this preliminary experiment showed the CD161 was expressed on a high number of lineage-CD127+cells in the PBMC of healthy controls (median=73.8%, IQR=61.5-85.7%). CD161 was expressed on a lower proportion of lineage-CD127+ cells in the SFMC (median=41.1%, IQR=37.2-49.3%) (Figure 4.2B). The scatter profiles of the CD161+ ILC populations were then investigated. CD161+ ILC from PBMC has a scatter profile consistent with lymphoid cells. In the SFMC CD161+ ILC had a mixed scatter profile (Figure 4.2A bottom row). Informed by these preliminary experiments, CD161 was added to the ILC panel for all subsequent analyses.

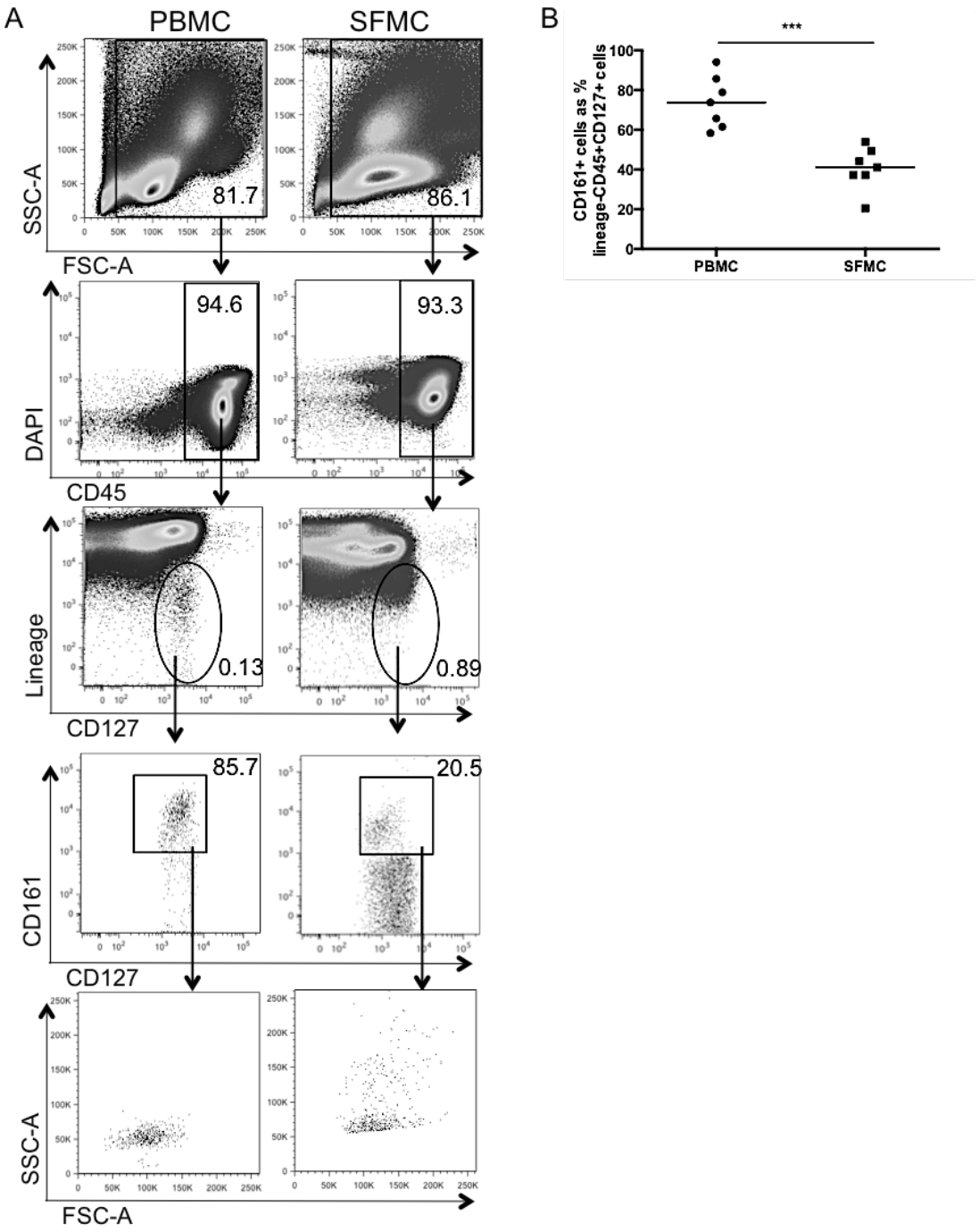


Figure 4.2 CD161 is useful for the identification of ILC in SFMC.

(A) Representative flow cytometry plots showing adapted ILC gating strategy including CD45 and CD161 in aHC PBMC (left panels) and SFMC from JIA patients (right panels). ILC populations were investigated within the total cell gate (top row) dead cells and doublets were excluded (data not shown). Leukocytes were selected based on expression of CD45 (second row) and CD161 expression was analysed on lineage-CD127+ cells (third and fourth rows). Bottom line shows back-gating analysis to show the size and granularity of CD161+/- cells from within the lineage-CD45+CD127+ population (B) Combined data showing proportion of CD161+ cells as a % of total lineage-CD45+CD127+ cells from within the PBMC of healthy controls (n=7) and SFMC of JIA patients (n=7). Statistical analysis by Mann- Whitney. p=0.0006

4.2.2 Group 3 ILC are enriched in the synovial fluid of JIA patients.

Following successful optimisation, ILC (lineage-CD45+CD127+CD161+) were enumerated within the PBMC and SFMC of JIA patients, and the PBMC of adult and child healthy controls (Figure 4.3A). The ILC population was small, as a proportion of all live mononuclear cells, in both PBMC and SFMC samples (0.005-0.5% of total live leukocytes). These data are consistent with other reports of the typical population frequency of ILC within human adult PBMC (Hazenberg and Spits, 2014a). No significant difference in the proportion of all live mononuclear cells identified as ILC by this strategy was seen between healthy or disease PBMC samples or SFMC (Figure 4.3B). Additionally, no differences were seen between ILC, as a proportion of all live mononuclear cells, between JIA subtypes analysed, namely, enthesitis-related arthritis, oligoarticular, polyarticular, and psoriatic arthritis (Figure 4.3C).

Based upon available literature at the time of these analyses, ILC were subsequently divided into subpopulations of ILC, defined according to expression of surface markers CRTH2, cKit and NKp44. ILC1 were defined as ILC which lacked expression of both CRTH2 and cKit; ILC2 were defined by expression of CRTH2; and ILC3 were identified as CRTH2 negative cells which expressed cKit, and could be further divided into NCR+ and NCR- groups based on expression of NKp44 (Figure 4.4A)(Mjosberg et al., 2011a, Hazenberg and Spits, 2014a).

These ILC subpopulations were then analysed as proportions of total ILC. This showed that ILC1 cells were significantly enriched, as proportion of all ILC, in the SFMC cells of JIA patients (median= 24.45%, IQR=16.63-37.3%) compared to patient or control PBMC (JIA PBMC median= 10.2%, IQR=8.67-14.2%) ($p>0.0001$) (Figure 4.4B). ILC2 were the most prevalent ILC population, as proportion of all ILC, in all PBMC samples analysed (median ILC2 in JIA PBMC= 60.8%, IQR=48.2-70.1%). In contrast a significant depletion of ILC2, as proportion of all ILC, was observed across SFMC samples (median= 7.7%, IQR=3.89-13.33%)($p<0.0001$) compared to PBMC of patients or controls (Figure 4.4C). NCR- ILC3 were significantly enriched, as a proportion of total

ILC in the SFMC of JIA patients (median = 51.1%, IQR=42.6-60.38%) compared to PBMC of patients or controls (median JIA PBMC NCR-ILC3= 29%, IQR=20.0-34.4%) ($p<0.0001$) (Figure 4.4D). Analysis of ILC3 expressing NKp44 revealed the emergence of an NCR+ILC3 population in the JIA SFMC cells (median= 8.44%, IQR=6.2-15.8%), which was significantly different to PBMC; I observed a near absence of this population in the PBMC samples of either patients or controls (median NCR+ILC3 in JIA PBMC= 0.25%, IQR=0-0.71%).

Although significant differences in ILC subpopulations were observed between PBMC and SFMC samples, no significant differences were seen between relative proportions of ILC subpopulations between the PBMC of adult and child controls or patient PBMC. Furthermore, no differences were seen in relative proportions of ILC populations between JIA clinical subtypes in the SFMC (Figure 4.4F-I). Although small numbers of patients were analysed for some clinical subtypes, given that the distinct pattern of relative proportions within SFMC compared to JIA PBMC was the same across all subtypes, in subsequent ILC investigations, JIA subtypes were grouped together and not analysed individually.

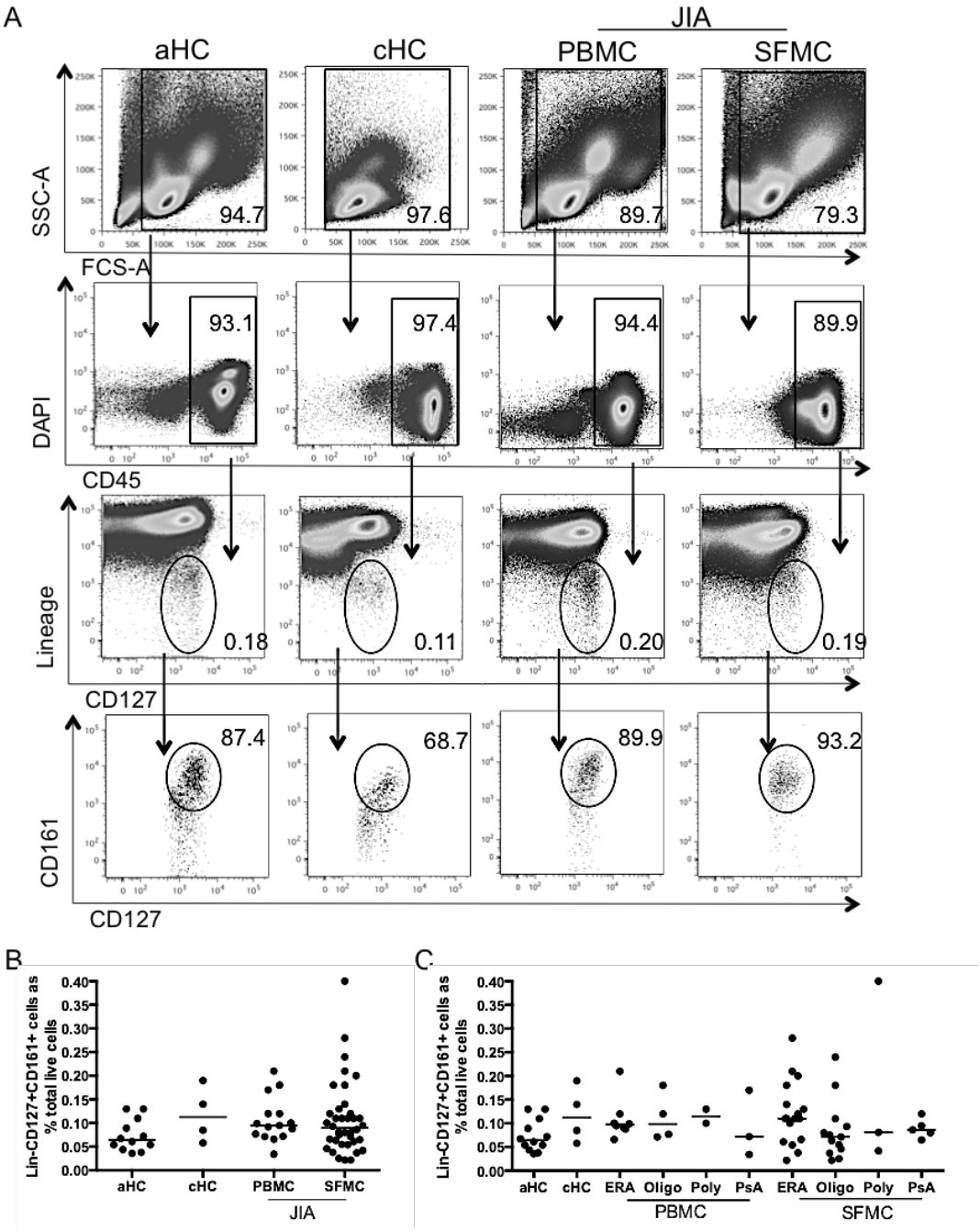


Figure 4.3 Enumeration of total ILC from the blood and synovial fluid of JIA patients and healthy controls.

Mononuclear cells were stained by multi colour flow cytometry for cell surface proteins to identify ILC populations. (A) Representative plots showing optimised gating strategy for identification of ILC ex vivo in PBMC from aHC and cHC and PBMC and SFMC from JIA patients. ILC were identified within gated total cells based on FSC-A and SSC-A (top row) and then as CD45+ leukocytes (second row) which are lineage- CD127+ (third row) and express CD161 on analysis (fourth row); (B) Combined data showing enumeration of ILC as % of total live CD45+ cells within PBMC of aHC (n=12) and cHC (n=4) controls, and blood PBMC (n=15) and joint SFMC (n=33) of JIA patients ex vivo; (C) Summary plot of enumeration of ILC defined as CD45+Lineage-CD127+CD161+ cells within PBMC of healthy adult and child controls (n=12, n=4 respectively) and PBMC and SFMC from ERA-JIA (n=8, n=17), oligo-JIA (PBMC n=4, SFMC n=13) poly-JIA (n=2,n=3) and PsA-JIA patients (n=3, n=5), shown as a percentage of total live mononuclear CD45+ cells. Statistical analysis by Kruskal Wallis. Bars represent median values.

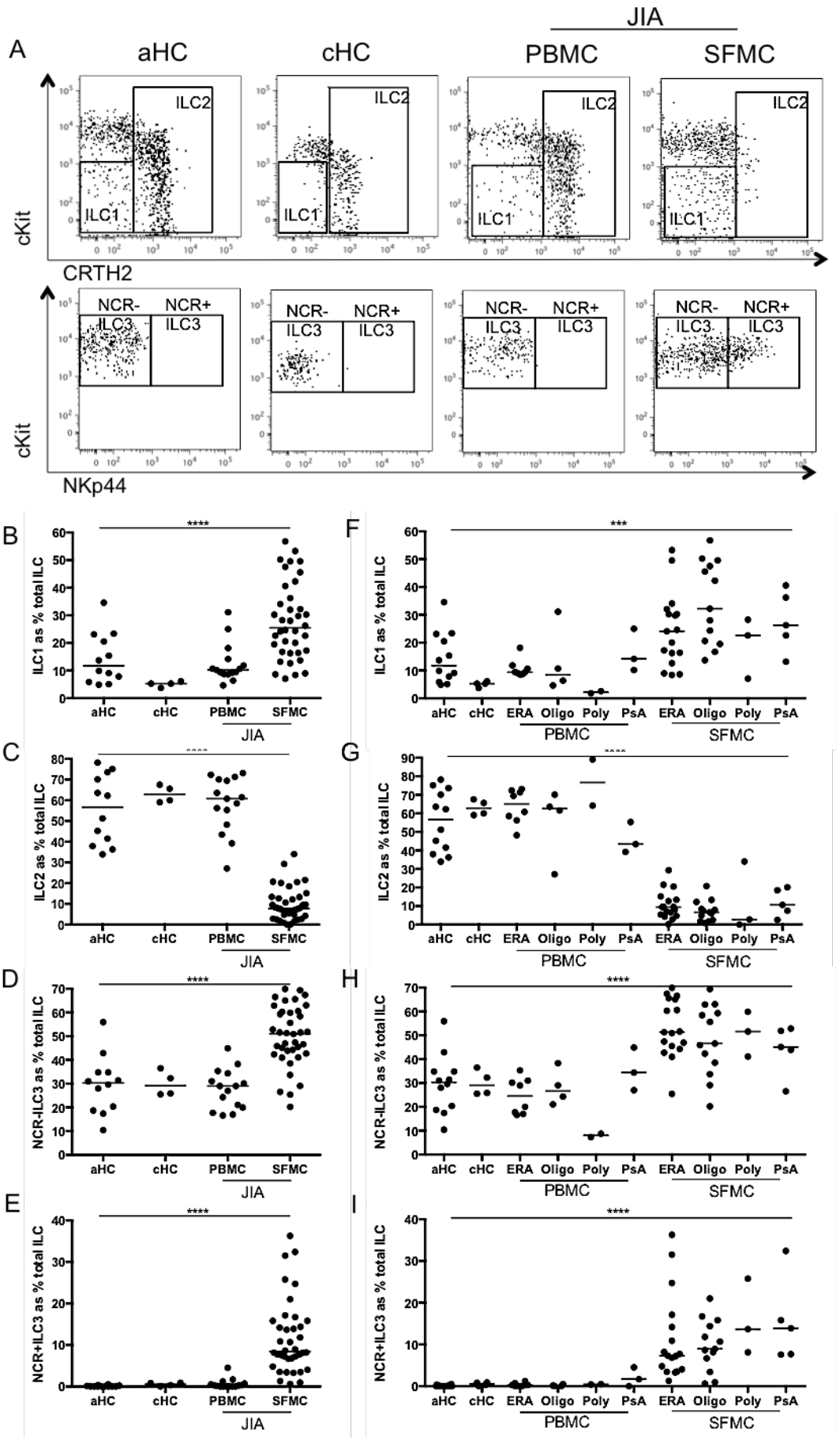

A
aHC
cHC
JIA
PBMC
SFMC
ILC2
ILC1
cKit
CRTH2
NCR-
NCR+
ILC3
NKp44
B
F
C
G
D
H
E
I
ILC1 as % total ILC
ILC2 as % total ILC
NCR-ILC3 as % total ILC
NCR+ILC3 as % total ILC
****
***
aHC
cHC
PBMC
SFMC
JIA
ERA
Oligo
Poly
PsA

Figure 4.4 ILC1 and ILC3 subpopulations are significantly enriched in the synovial fluid mononuclear cells of JIA patients.

ILC subpopulations were identified by multicolour flow cytometry within control PBMC from aHC and cHC. (n=12, n=4) and PBMC and SFMC from ERA-JIA (n=8, n=17) oligo-JIA (n=4, n=13) poly-JIA (n=2,n=3) and PsA patients (n=3, n=5). (A) Representative plots showing the gating strategy for the identification of ILC subpopulations, (ILC1, ILC2, NCR-ILC3 and NCR+ILC3) within the total ILC population identified ex vivo (gating as shown in Figure 4.3) in PBMC from aHC and cHC and PBMC and SFMC from JIA patients. Subpopulations defined according to phenotype: ILC1 (CRTH2-cKit-) ILC2 (CRTH2+) NCR-ILC3 (CRTH2-cKit+NKp44-) and NCR+ILC3 (CRTH2-cKit+NKp44+); (B-E): Summary data showing frequency of (B) ILC1 (C) ILC2 (D) NCR-ILC3 and (E) NCR+ILC3, each as a percentage of total ILC within healthy control PBMC (aHC n=12, cHC n=4) and JIA PBMC (n=15) and SFMC (n=33); (F-I): Combined data summary plots enumerating of ILC subpopulations as % of total ILC, within PBMC of adult (aHC, n=12) and child (cHC n=4) controls, and blood PBMC (n=15) and joint SFMC (n=33) of JIA patients divided by clinical JIA subtype ex vivo. (F) ILC1 (G) ILC2 (H) NCR-ILC3 and (I) NCR+ILC3. Statistical analysis carried out by Kruskal Wallis. *p<0.05 **p<0.01 ***p<0.001 ****p<0.0001. Bars represent median values.

4.2.3 Multispectral imaging flow cytometric analysis of paired blood and synovial fluid ILC confirms lymphoid cell morphology

Multispectral imaging flow cytometric analysis (ImageStream) was used to visualise ILC subpopulations from the PBMC and SFMC of JIA patients, and confirm membrane staining of the cell surface proteins. Cells were selected based on aspect ratio intensity and area measurements, and focused cells (Gradient RMS>50) were selected and analysed using the optimised ILC panel in order to identify lineage-CD45+CD127+CD161+ ILC (Figure 4.5A).

Multispectral images of ILC subsets were generated by ImageStream software and analysed for cell morphology and cell marker localization. ILC cell markers were restricted to the cell surface. Additionally, the cells showed very little fluorescence in the side scatter plots supporting a lymphocyte phenotype. ILC from PBMC and SFMC show lymphoid cell morphology. All ILC detected appeared to have a characteristically dense single nuclei and thin halo of perinuclear cytoplasm. Additionally, although ILC from the SFMC appear to be slightly larger than parallel PBMC ILC, cells from both compartments measured ~7µm, the approximate size expected for lymphocytes, (Figure 4.5B and 4.5C, left hand of each set of panel).

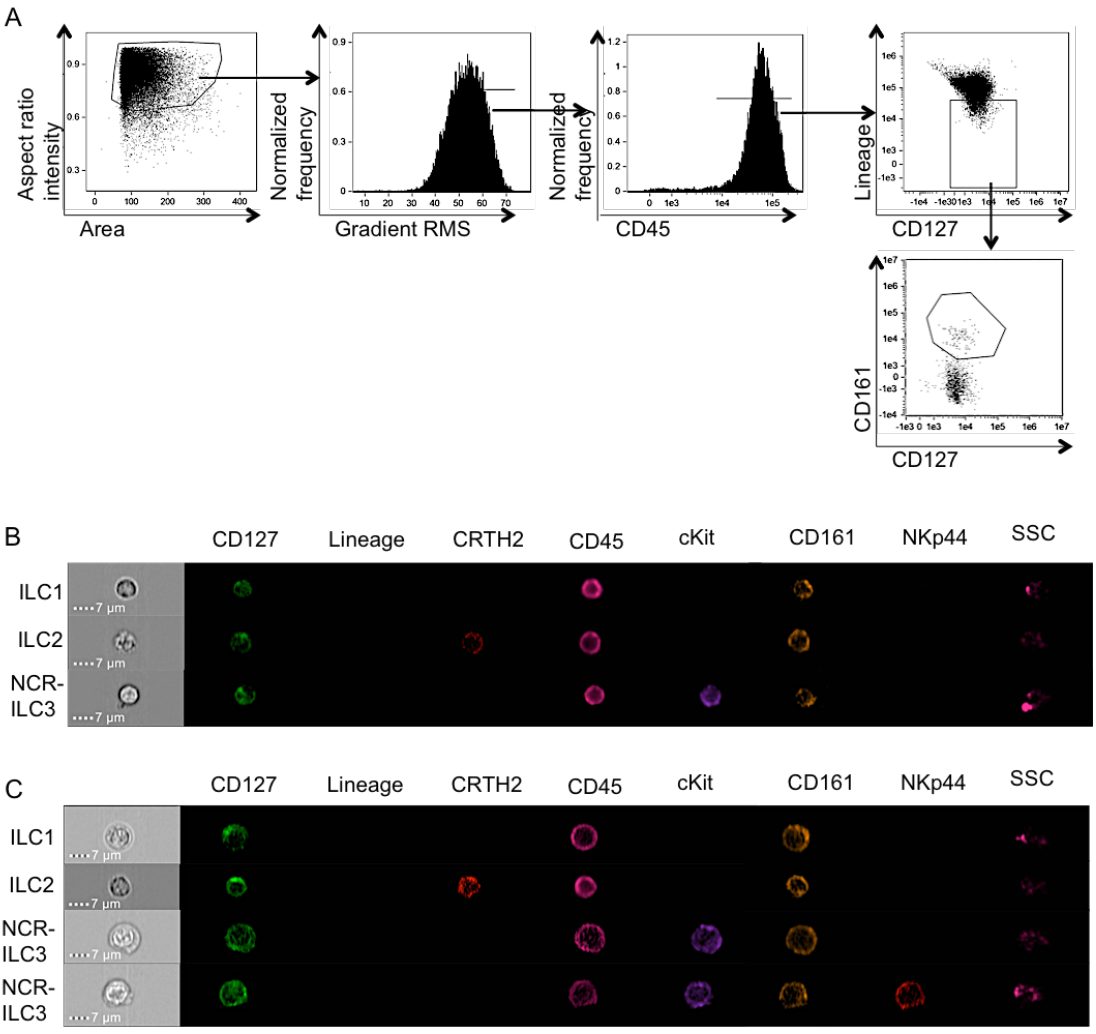


Figure 4.5 ImageStream analysis of ILC populations confirms lymphoid cell morphology and surface protein expression.

Paired PBMC and SFMC from JIA patients were analysed by Image Stream ex vivo after depletion of T cells by removal of CD3+ cells using magnetic beads. (A) Image stream gating strategy for identification of ILC populations. Cells were identified according to aspect ratio intensity and area. Focused cells were selected with Gradient RMS>50; ILC were identified as Lineage- CD45+CD127+CD161+ cells; (B, C) ILC subpopulations were identified according to fluorescence of subset specific protein expression by analysis of individual cell images as shown. Representative images of ILC subpopulations from (B) PBMC and (C) SFMC from JIA patients. Left hand panel (grey) in each panel shows the brightfield image of the cell.

4.2.4 High purity ILC can be sorted by flow cytometry from mononuclear cells prepared from blood and synovial fluid

In order to validate the analysis of ILC identified in PBMC and SFMC, I investigated transcription factor and cytokine expression profiles of ILC. Total ILC (lineage-CD45+CD127+CD161+) were sorted by flow cytometry from aHC PBMC and JIA SFMC samples following depletion of T cells by CD3 immuno-magnetic selection. To ensure that the sorting procedure did not affect ILC populations, or the expression of crucial surface markers (CRTH2, cKit and NKp44), a sample was removed after each stage of the sorting process and stained with the full ILC subtyping panel. The sorting process did not affect ILC subpopulations as shown in Figure 4.6B. ILC were sorted according to the sorting strategy shown in Figure 4.6A and highly pure samples were isolated from each sort from both PBMC and SFMC (>92% pure) (Figure 4.6C and summary of sort purity data, 4.6D).

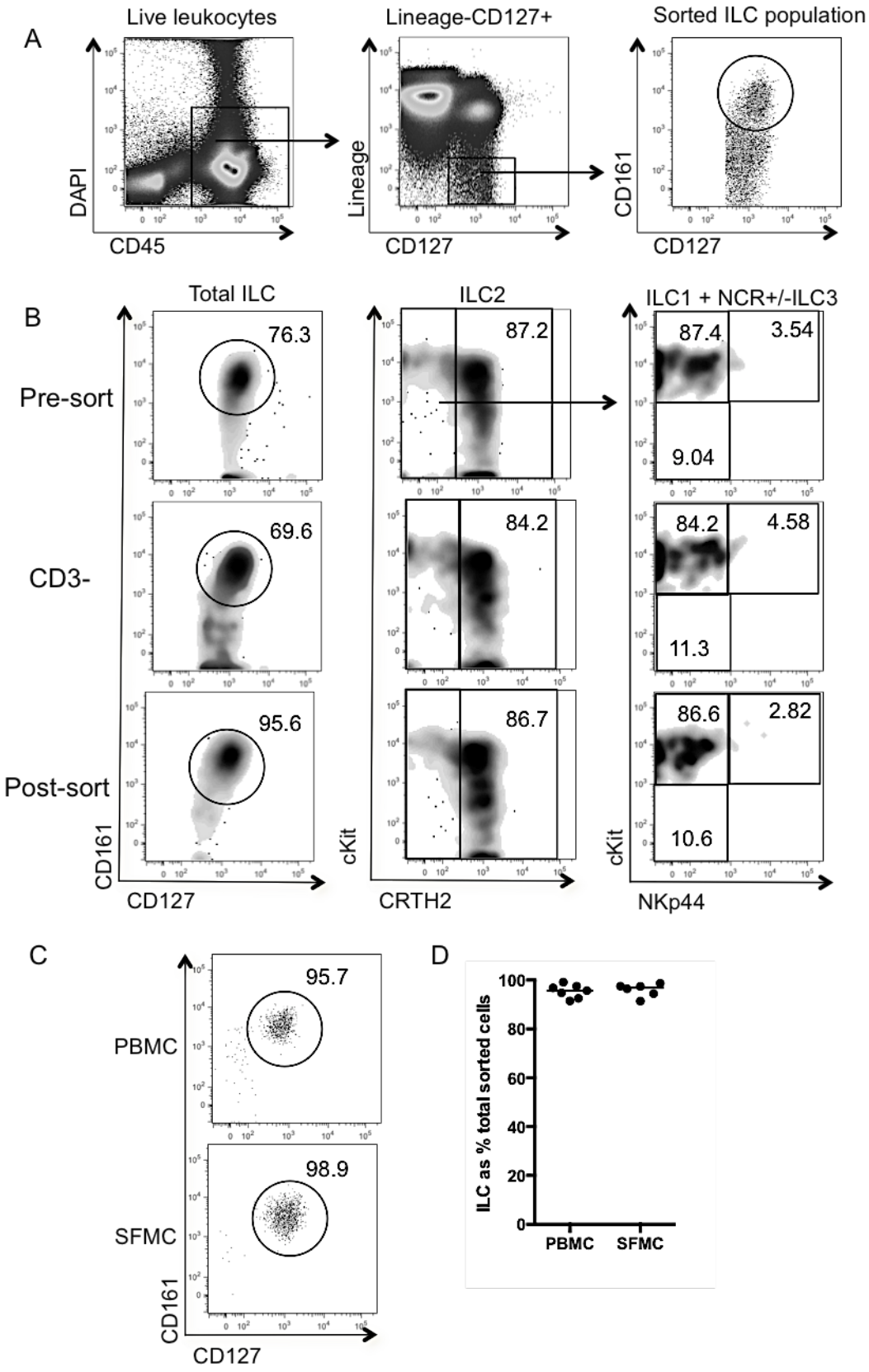

A
Live leukocytes
Lineage-CD127+
Sorted ILC population
DAPI
CD45
Lineage
CD127
CD161
CD127
B
Total ILC
ILC2
ILC1 + NCR+/-ILC3
Pre-sort
CD3-
Post-sort
76.3
69.6
95.6
87.2
84.2
86.7
87.4
3.54
9.04
84.2
4.58
11.3
86.6
2.82
10.6
CD161
CD127
cKit
CRTH2
cKit
NKp44
C
PBMC
SFMC
95.7
98.9
CD161
CD127
D
ILC as % total sorted cells
100
80
60
40
20
0
PBMC
SFMC

Figure 4.6 Validation of strategy for sorting ILC.

Total ILC (lineage-CD45+CD127+CD161+) were sorted by flow cytometry from healthy control PBMC (n=7) and JIA SFMC (n=6) (A) Sorting strategy based on expression of CD45, lineage markers, CD127 and CD161; (B) ILC from healthy PBMC analysed for ILC subpopulation markers, before sorting (top row, pre-sort), after CD3 depletion by magnetic beads (middle row, CD3-), and after sorting (bottom row, post-sort), as shown, to verify that sorting process did not affect critical marker expression. Total ILC (left plot) were split into CRTH2+ (ILC2) and CRTH2- populations (middle plot) and subsequently the CRTH2- cells were divided into ILC1 (cKit-NKp44-), NCR-ILC3 (cKit+NKp44) and NCR+ILC3 (cKit+NKp44+) subpopulations (right plot); (C) Representative dot plots showing purity of ILC (based on CD161+ CD127+) isolated from healthy PBMC and the SFMC of JIA (ERA subtype) patients, following exclusion of dead cells; (D) Summary plot showing sort purities from sorting of ILC from PBMC and SFMC, defined as % live cells that were CD45+lineage-CD127+CD161+, post sorting.

4.2.5 qPCR analysis of sorted ILC show altered transcription factor and cytokine profiles between blood and synovial fluid

The transcription factor and cytokine profiles of ILC (lineage-CD45+CD127+CD161+) were analysed for specific transcripts by qPCR. Gene expression of transcription factors *TBX21* (Tbet) (ILC1), *GATA3* (ILC2)*, RORC* (ILC3) and *AHR* (ILC3) were analysed in FACS-sorted ILC from healthy PBMC and SFMC from JIA patients. Data were expressed as relative expression compared to gene expression from skewed CD4+ T cells prepared and analysed as detailed in chapter 2, and normalized to β-actin expression (*ACTB*). Relative expression of *GATA3* was higher in ILC isolated from healthy PBMC samples compared to those sorted form SFMC. Conversely, expression of *TBX21, RORC* and *AHR* were all increased in SFMC relative to healthy PBMC (Figure 4.7).

In parallel, expression of signature cytokine genes *IFNG (*IFNγ*), IL13, IL17A* and *IL22* were analysed. Higher expression of *IFNG, IL17A* and *IL22* were detected in SFMC ILC, while *IL13* expression was increased in ILC isolated from healthy control PBMC samples (Figure 4.8).

Taken together, these qPCR data show that the relative proportions of ILC in SFMC from JIA patients compared to healthy PBMC are mirrored in the ratio of gene expression of signature transcription factors and cytokines in synovial: blood ILC. The ILC subpopulation proportions and the qPCR data were then analysed as ratios, comparing the data from blood ILC of aHC to synovial fluid ILC. In the SFMC I observed an enrichment of ILC1 and ILC3 populations, and in parallel, increased expression of transcripts for the related genes (*TXB21, RORC, AHR, IFNG, IL17A* and *IL22)* compared to PBMC ILC. On the other hand I observed a relative depletion of ILC2 in the SFMC, and reduced *GATA3* and *IL13* gene expression compared to expression in ILC from PBMC (Figure 4.9).

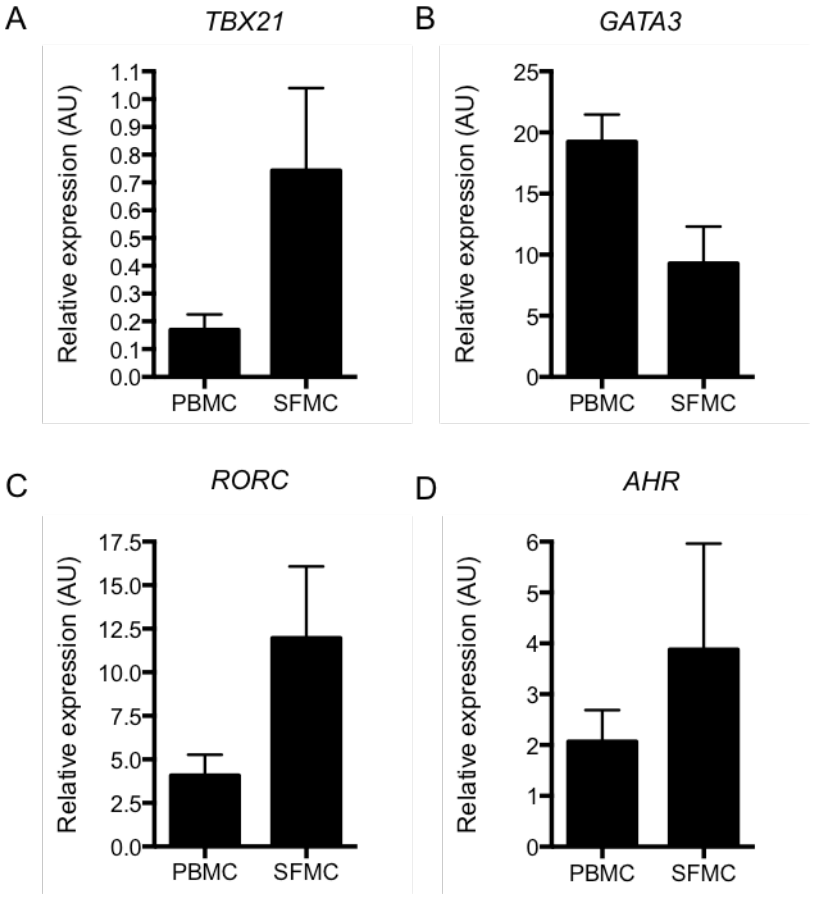


Figure 4.7 Relative expression of mRNA transcripts for master transcription factor genes in ILC.

qPCR was performed to amplify specific transcripts from cDNA generated from ILC sorted from aHC PBMC (n=3) and JIA SFMC (n=3). Relative expression of specific transcripts for (A) *TXB21* (B) *GATA3* (C) *RORC* (D) *AHR*. Bars represent the mean value of the 3 ILC samples, error bars represent SEM.

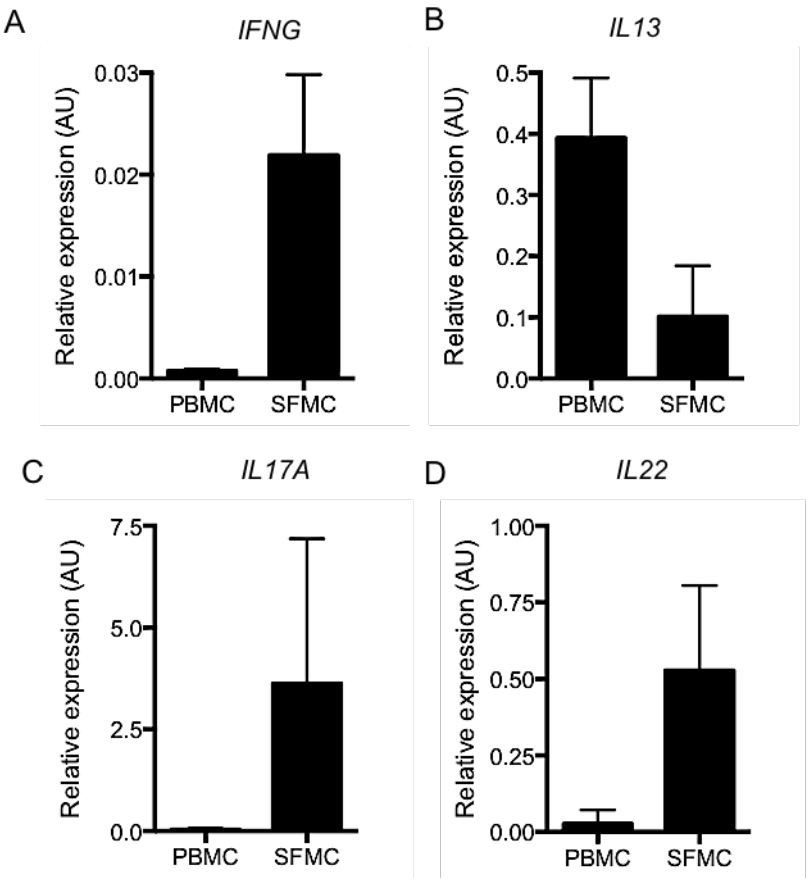


Figure 4.8 Relative expression of mRNA transcripts for signature cytokine genes in ILC.

qPCR was performed to amplify specific transcripts from cDNA generated from ILC sorted from aHC PBMC (n=3) and JIA SFMC (n=3). Relative expression of specific transcripts for (A) *IFNG* (B) *IL13* (C) *IL17A* (D) *IL22*. Bars represent the mean value of the 3 ILC samples, error bars represent SEM.

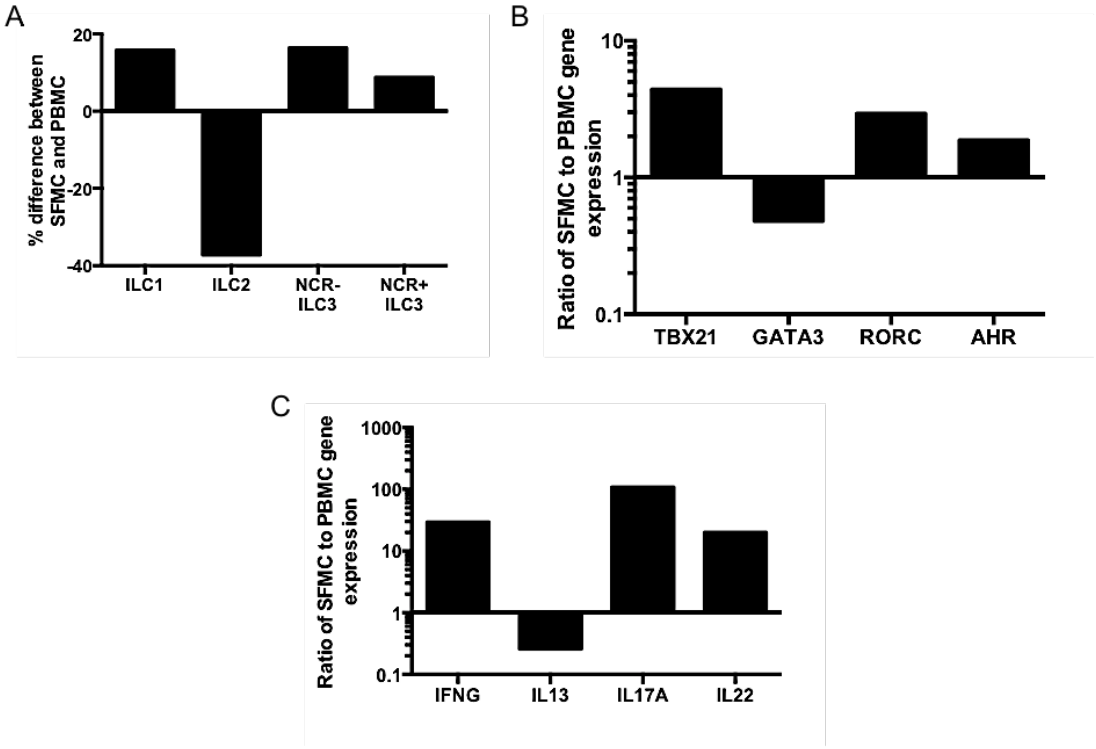


Figure 4.9 Comparison of ILC proportions analysed by flow cytometry and expression of transcription factors and cytokine transcripts, analysed by qPCR, in healthy PBMC and JIA SFMC.

(A) Relative difference between mean proportions of ILC cell types (ILC1, ILC2, NCR-ILC3 and NCR+ILC3) in healthy PBMC and JIA SFMC identified by flow cytometry (data from Figure 4.3); (B) Ratio of mean gene expression of signature transcription factors in SFMC: PBMC (data from Figure 4.7). (C) Ratio of mean gene expression of signature cytokines in SFMC: PBMC (qPCR), (data from Figure 4.8).

4.2.6 Synovial ILC produce reduced IL-13 *ex vivo*

In order to interrogate the functionality of ILC, mononuclear cells from patient PBMC and SFMC, and aHC PBMC were stimulated for 4 hours with PMA and Ionomycin in the presence of Brefeldin A and signature cytokines IFNγ, IL-13, IL-17A and IL-22 were measured by intracellular staining by flow cytometry. The gating strategy for ILC is shown in Figure 4.3, and for subsequent analysis of cytokine positive cells, as shown in Figure 4.10A. Low levels of IFNγ, IL-17A and IL-22 were observed in all samples, with only slight trend to more of these cytokines being produces by ILC from SFMC. There were no significant differences in cytokine positive cells seen between PBMC and SFMC ILC for IFNγ, IL-17A and IL-22 (Figure 4.10B, 4.10D and 4.10E).

A significant reduction in IL-13 production was seen in synovial ILC (median= 7.53%, IQR=2.45-18.65%) compared to JIA PBMC (median= 39.95%, IQR=22.78-51.2%) (Figure 4.10C). This result correlated well with the observed significant reduction in ILC2 ($p=0.019$), as proportion of total ILC2 population within SFMC. As the number of samples for these analyses was small the results may be relatively underpowered and more significant differences may have been seen with larger sample numbers.

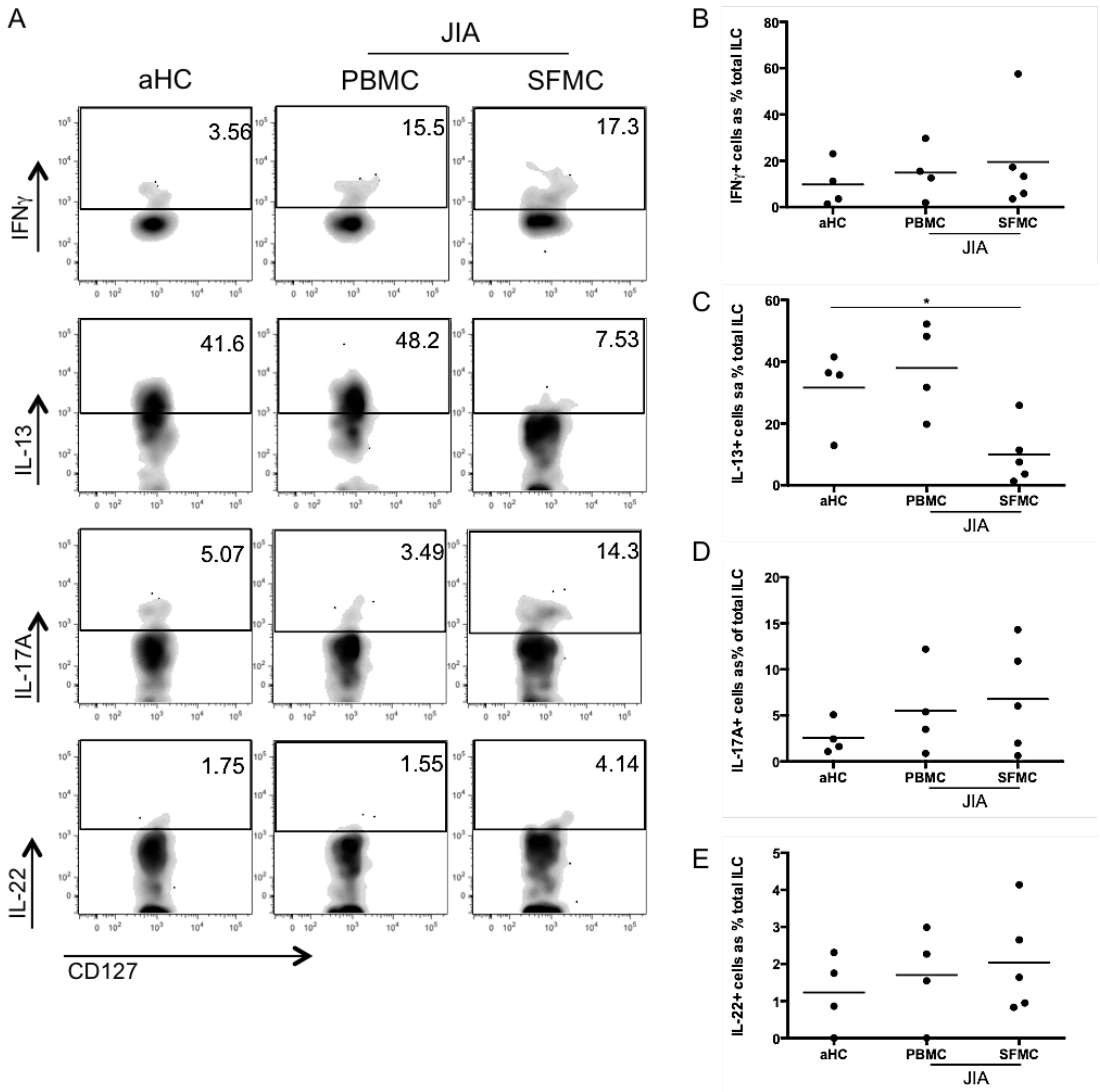


Figure 4.10 Cytokine production by ILC analysed ex vivo upon stimulation.

PBMC from blood of aHC (n=4) and JIA patients (n=4), and SFMC from JIA patients (n=5) were analysed for cytokine production by flow cytometry following 4 hours stimulation with PMA and Ionomycin in the presence of Brefeldin A. Cells were first gated on total ILC as shown in figure 4.3 and subsequently analysed for each cytokine (A) Representative flow cytometry plots showing IFNγ (top row), IL-13 (second row), IL-17A (third row) and IL-22 (bottom row) in blood of aHC (left column), JIA PBMC (middle column) and SFMC of JIA patients (right column). Summary plots showing proportion of ILC producing (B) IFNγ (C) IL-13 (D) IL-17A and (E) IL-22 within each sample. Line represents median value for each data set. Statistical analysis carried out by Kruskal Wallis. *p<0.05.

4.2.7 FACS sorted synovial ILC produce inflammatory cytokines in response to cytokine stimulation

ILC are known to potently produce cytokines in response to the surrounding cytokine milieu. In order to investigate the response of ILC from aHC PBMC and JIA SFMC to activating cytokines, ILC were sorted by flow cytometry as detailed in section 4.2.4 and Figure 4.6. Sorted pure ILC were stimulated for 4 days in the presence of IL-2 and cytokine cocktails designed to activate ILC1, ILC2 or ILC3 populations as detailed in chapter 2. All cultures were supplemented with IL-2 plus: for ILC1 addition of IL-12 and IL-18; for ILC2 addition of TSLP, IL-25 and IL-33; for ILC3 addition of IL-1β and IL-23. For the final 4 hours of culture, the cells were stimulated with PMA and Ionomycin in the presence of Brefeldin A.

Under ILC1-activating conditions (IL-12 and IL-18) synovial ILC potently produced IFNγ (median= 51.25%, IQR=43.0-59.5%), while only a small proportion of blood ILC were observed to produce the cytokine (median= 15.20%, IQR=11.61-30.3%), Figure 4.11A and 4.11B. Under ILC2-activating conditions (TSLP, IL-25 and IL-33) IL-13 production was induced in a significant proportion of blood ILC (median= 62.95%, IQR=51.45-68.08%) while synovial ILC showed a smaller proportion of cells able to produce IL-13 (median = 18.30%, IQR=13.2-23.4%) Figure 4.11A and 4.11C. Finally, under ILC3-activating conditions (IL-23 and IFNβ), little IL-17A production was seen from either blood or synovial ILC (median= 3.64%, IQR=2.67-4.09 and median=3.38%, IQR=2.48-4.7% respectively) Figure 4.11A and 4.11D. However, increased IL-22 production was seen from synovial ILC (median=9.17%, IQR=6.04-12.3%) compared to aHC blood ILC (median= 3.2%, IQR=2.2-3.88%) (Figure 4.11A and 4.11E).

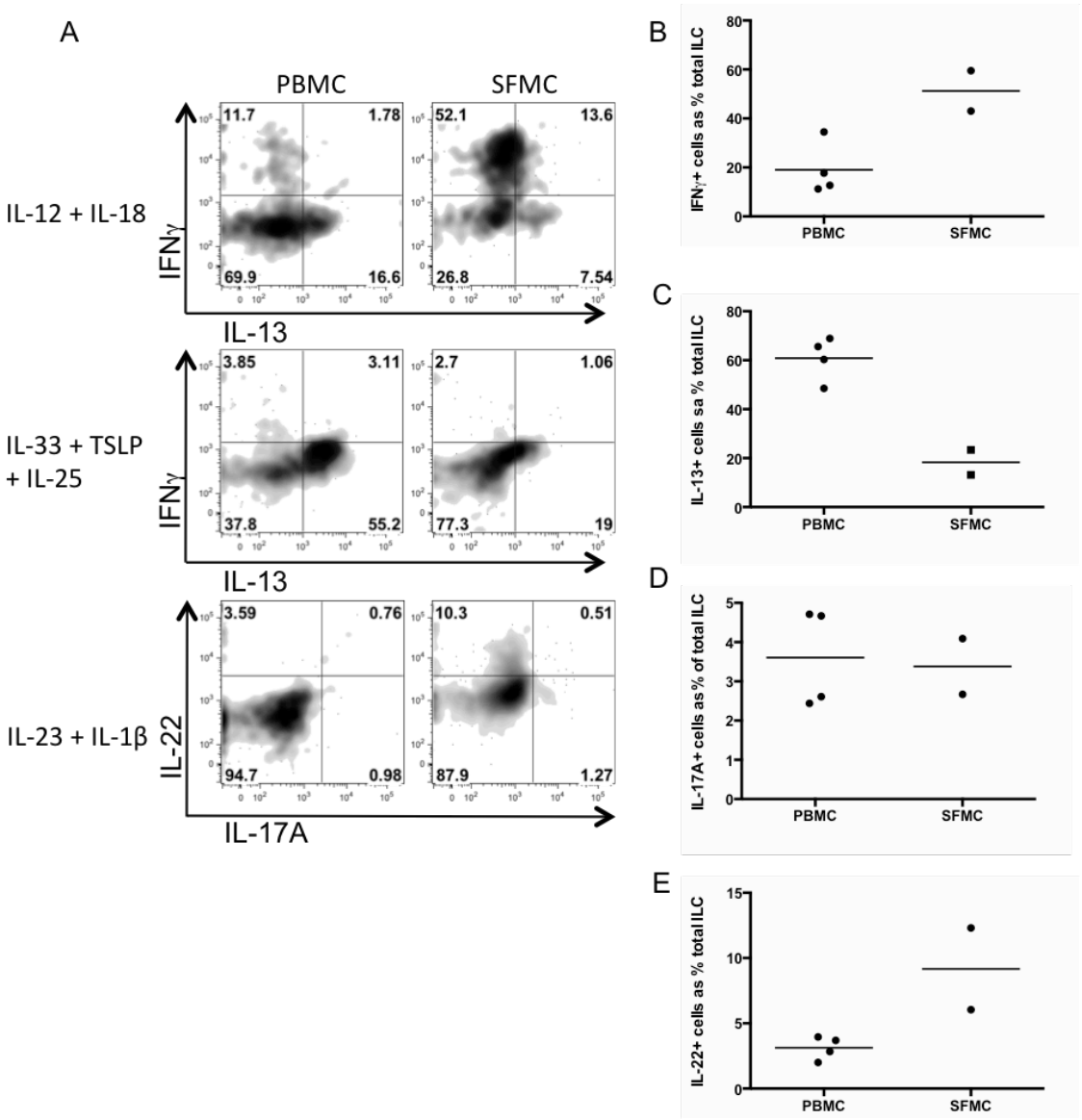


Figure 4.11 Cytokine production by cultured ILC.

Total ILC were sorted from healthy PBMC (n=5) and JIA SFMC (n=2), cultured for 4 days in the presence of IL-2 plus skewing cytokines as follows: IL-12 and IL-18 for ILC1, IL-33, TSLP and IL-25 for ILC2, and IL-23 and IL-1β for ILC3 and then stimulated for 4 hours with PMA and Ionomycin in the presence of Brefeldin A. (A) Representative flow cytometry plots showing production of IFNγ, IL-13, IL-17 and IL-22; (B) summary data for IFNγ (C) IL-13 (D) IL-17A and (E) IL-22 cytokine production from synovial and blood ILC. Lines represent median value for each data set.

4.2.8 CD127 and CD161 are down regulated upon activation of ILC in vitro

ILC sorted form healthy PBMC that were cultured with stimulating cytokines (as detailed in Figure 4.11) grew larger as they became activated, which could be detected as the forward and side scatter profile of the ILC transformed during the course of the stimulation assay (Figure 4.12A). When these cells were analysed on day 4 of the culture assays, expression of CD127 and CD161 was significantly lower on the activated ILC compared to unstimulated control ILC from the same aHC. This is demonstrated by the >3 fold reduction in mean protein expression (analysed by MFI value) of both CD127 and CD161.

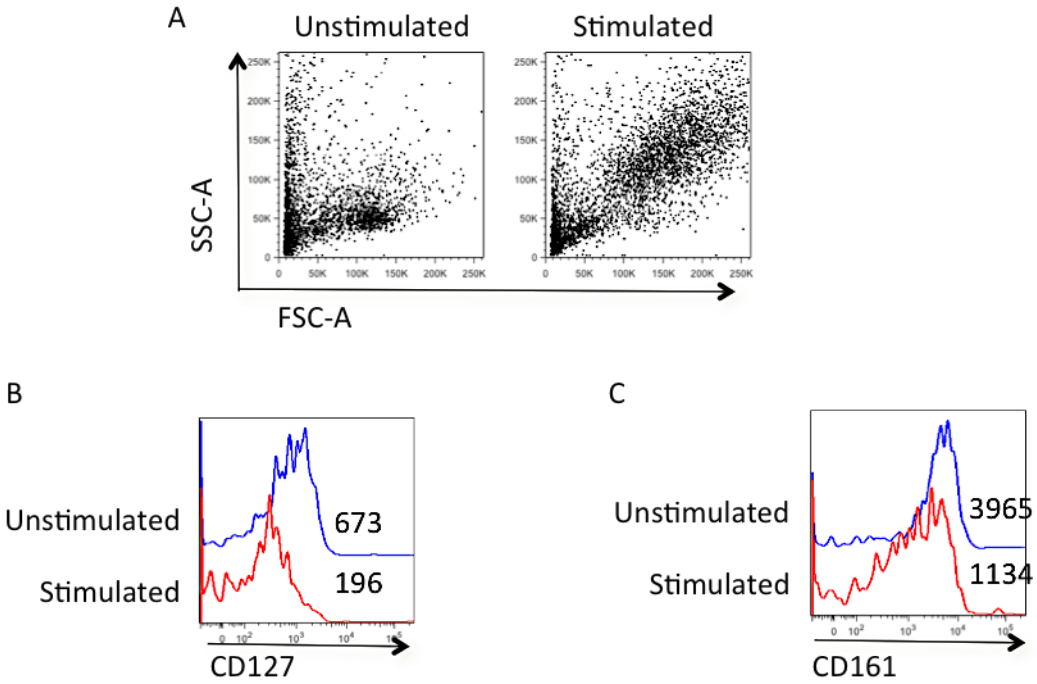


Figure 4.12 The effect of cytokine driven activation on ILC cell size and expression of CD127 and CD161.

(A) Representative dot plot showing increase in size and granularity of ILC after 4 days culture with activating cytokines IL-2, IL-25, IL-33 and TSLP. Cells were analysed for expression of CD127 and CD161 by flow cytometry; (B) CD127 expression on ILC before (blue line) and after (red line) cytokine stimulation; (C) CD161 expression on synovial ILC before (blue line) and after (red line) cytokine stimulation. Plots in B and C are gated on live single cells. Figures represent MFI values for CD127 (B) and CD161(C).

4.2.9 Proportions of ILC populations change over time in the joints of JIA patients and are associated with disease status

To ascertain whether there was a time-dependent relationship between relative proportions of ILC subpopulations in disease, ILC were characterised within SFMC taken from 3 serial time points of 3 JIA patients. The medication status of the patients and physicians overall disease activity score, VAS (0-10 with 0 signifying no active disease, 10 worst disease activity), were collected for each patient at each time point (Table 4.2). Serial samples used during this preliminary investigation were collected from 3 were oligoarthritis patients. Since joint fluid is only aspirated for clinical indications, all patients from whom synovial fluid is collected have some on-going disease activity, as indicated by the physicians VAS scores, here ranging from 1.4 to 5.0.

The proportions of ILC2 as a percentage of total ILC remained relatively stable over time in all 3 patients and were consistently low in all samples (represented by blue lines in Figure 4.13). Relative proportions of ILC1 and NCR- and NCR+ ILC3 varied over time in all 3 patients. This preliminary investigation suggests that higher proportions of NCR-ILC3 were seen when patients had worse disease, as defined by higher physicians VAS. Conversely, both ILC1 and NCR+ILC3 proportions rose when NCR-ILC3 fell suggesting that the proportion of these ILC subpopulations, within SFMC may be linked to less severe disease as assessed according to the physicians VAS (Figure 4.13D, 4.13E, 4.13F and Table 4.1).

		Patient 1	Patient 2	Patient 3
Sample 1	Physicians VAS	1.0	4.0	5.0
	Medications	MTX	MTX	Nil
	Duration of disease (years)	11.2	6.5	1.1
Sample 2	Physicians VAS	4.0	2.0	1.7
	Medications	MTX	MTX	MTX
	Duration of disease (years)	11.9	10.8	4.7
Sample 3	Physicians VAS	2.0	1.7	1.4
	Medications	MTX	MTX	MTX
	Duration of disease (years)	14.4	11.5	7.25

Table 4.1 Physicians VAS scores, (range 0.0-10.0), medication and disease duration of the patients at each of the 3 time points of sampling

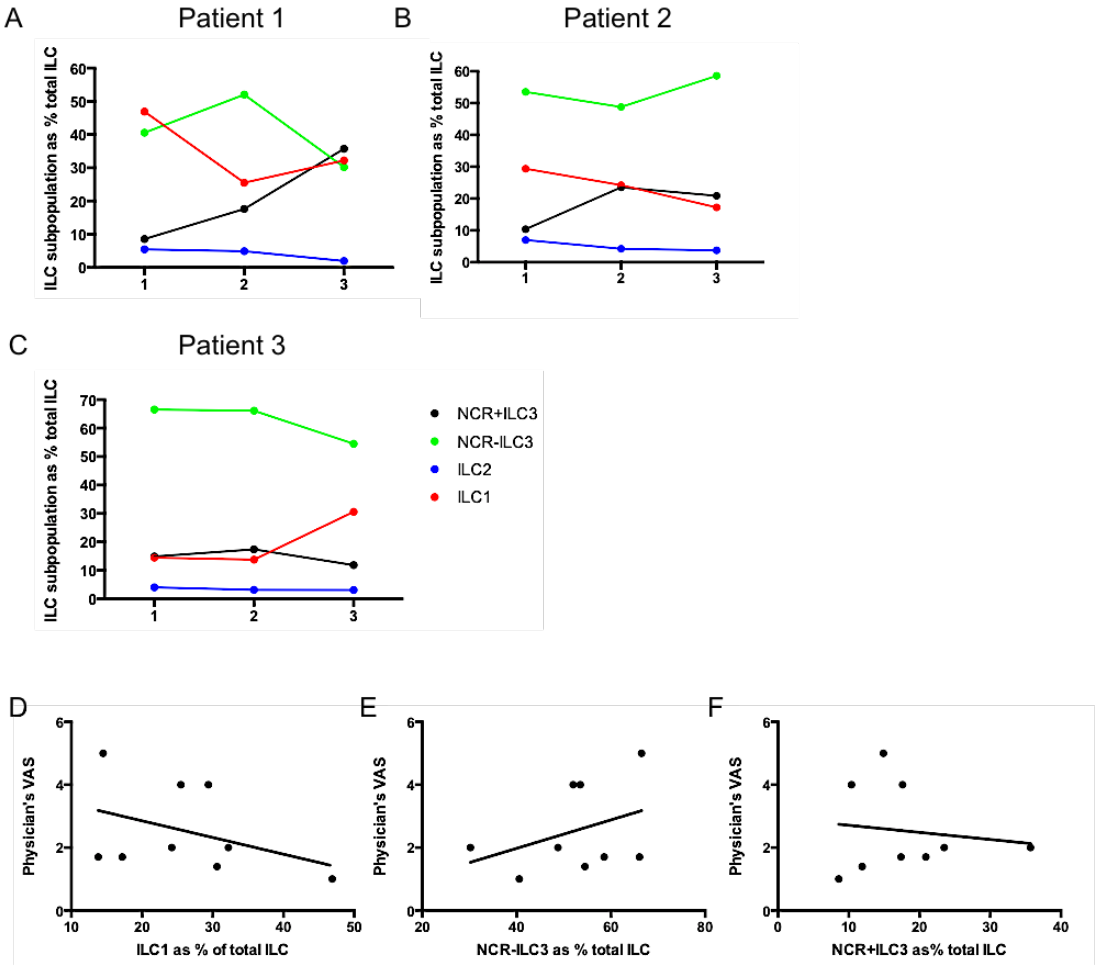


Figure 4.13 Proportions of ILC populations within SFMC from the inflamed joint of JIA patients fluctuate between serial clinical samples.

(A-C)Summary plots showing relative proportions of ILC subpopulations identified by surface marker expression (as defined in Figure 4.4) in serial SFMC samples collected from JIA patients (n=3) over several years. (D-F) Correlation analysis of ILC subpopulations in the SFMC against physician's VAS scores. Combined data from 3 serial clinical samples from 3 JIA patients. (D) ILC1 analysed against physicians VAS (E) NCR-ILC3 analysed against physicians VAS and (F) NCR+ILC3 analysed against physicians VAS.

4.2.10 Proportions of synovial NCR- and NCR+ ILC3 are inversely correlated with each other, and are associated with clinical measures of disease severity

Analysis of relationships between relative proportions of ILC subpopulations in the serial SFMC samples from JIA patients suggested that the NCR- and NCR+ ILC3 populations appeared to be inversely associated. To analyse this trend at a population level ILC3 subpopulations, as proportion of total ILC in SFMC, were correlated in a larger set of SFMC from 33 JIA patients. Analysis of the relative proportions of ILC subpopulations within the SFMC samples from the 33 JIA patients analysed here showed a weak but significant inverse correlation between NCR- and NCR+ ILC3 (r=-0.43, p=0.043) (Figure 4.14).

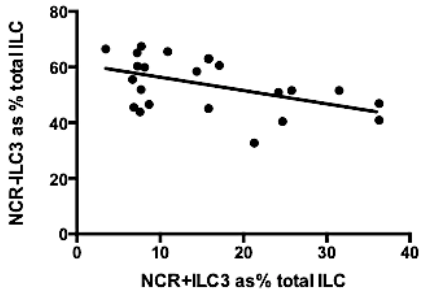


Figure 4.14 Proportions of NCR+ILC3 and NCR-ILC3 subpopulations within SFMC are inversely correlated.

Analysis of ILC subpopulations in SFMC of JIA patients n=33 *ex vivo*. Correlation analysis by Spearman correlation r=-0.43, p=0.043.

In order to explore the significance of the two distinct ILC3 subpopulations in relation to JIA disease severity, relative proportions of NCR- and NCR+ ILC3, here analysed as a proportion of total live cells, were analysed against measures of disease severity. For this analysis, active joint count, ESR and physicians VAS (0.0- 10.0) at time of joint sample were included, as well as the JADAS3 score (range 0-30). The JADAS3 is calculated as a composite score using 3 disease activity variables, namely the active joint count, physicians VAS and a parent global score of disease scored as a VAS (0.0- 10.0)(McErlane et al., 2013). Higher proportions of NCR-ILC3 were positively associated with the number of active joints affected at the time of synovial fluid sample collection ($r=0.55$, $p=0.0055$) (Figure 4.15A). The proportion of NCR-ILC3 was also weakly associated with ESR ($r=0.39$, $p=0.08$) and significantly correlated with the physician's VAS ($r=0.55$, $p=0.005$) and JADAS3 score ($r=0.75$, $p=0.004$) at the time of sample collection (Figure 4.15B, 4.15C, 4.15D). No association was found between NCR+ILC3 as a proportion of total live cells and active joint count (0.04, $p=0.85$), ESR (0.32, $p=0.15$) or JADAS3 score ($r=-0.38$, $p=0.20$) at the time of sample collection (Figure 4.15E, 4.15F and 4.15H). However, a significant negative correlation was found between NCR+ILC and physician's VAS ($r=-0.43$, $p=0.04$).

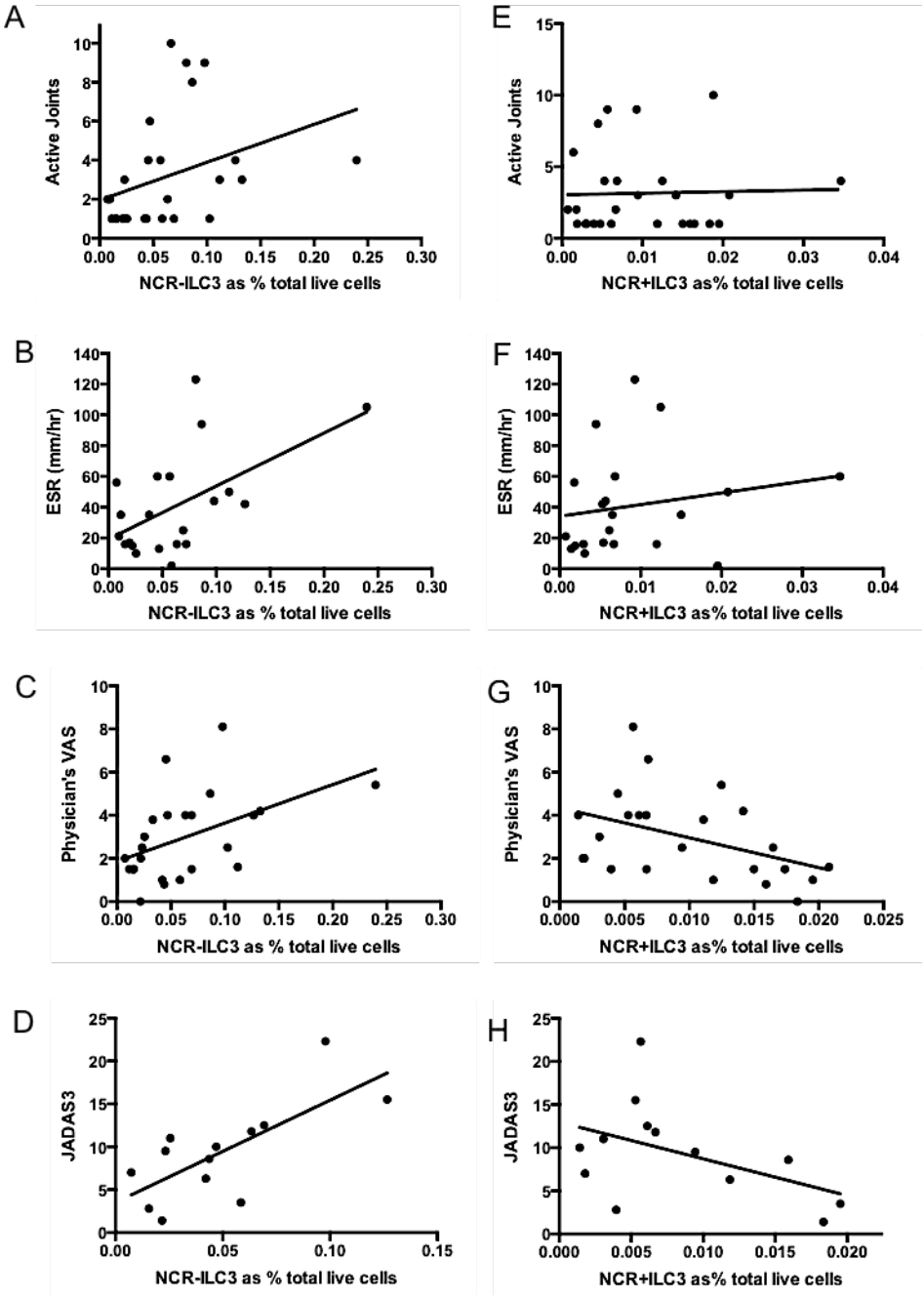


Figure 4.15 Proportion of IL-17-producing ILC3 in the SFMC correlate positively with disease severity

(A-H) Correlations in the SFMC of JIA patients between ILC subpopulations analysed as a % of total live cells and clinical measures of disease severity (A) NCR-ILC3 analysed against active joint count at time of sample (n=27, r=0.51, p=0.0055); (B) NCR-ILC3 analysed against ESR at time of sample (n=20, r=0.39, p=0.08); (C) NCR-ILC3 analysed against physician's VAS as time of sample (n=24, r=0.55, p=0.005); (D) NCR-ILC3 analysed against JADAS3 score as time of sample (n=13, r=0.75, p=0.004); (E) NCR+ILC3 analysed against active joints at the time of sample (n=27, r=0.04, p=0.85); (F) NCR+ILC3 and ESR at time of sample (n=21, r=0.32, p=0.15); (G) NCR+ILC3 analysed against physicians VAS (n=24, r=-0.43, p=0.04); (H) NCR+ILC3 analysed against JADAS3 score as time of sample (n=13, r=-0.38, p=0.20) Correlation analysis by Spearman correlation.

4.2.11 Synovial ILC3 correlate with inflammatory IL-17+ CD4, CD8 cells, and regulatory CD4 T cells.

Correlation analysis was carried out on ILC3 subpopulations, IL-17A-producing CD4, CD8 and CD4-CD8- T cell subpopulations and Foxp3-ve CD4 Treg within mononuclear cell populations of the PBMC and SFMC of JIA patients in order to elucidate associations between ILC and T cell subtypes.

Significant positive correlations were seen in SFMC between NCR-ILC3 as a proportion of total live cells, and IL-17A-positive cells in the CD4+ ($r=0.49$, $p=0.042$) and CD8+ ($r=0.59$, $p=0.011$) T cell compartments, a trend towards association with γδ (defined as CD4-CD8- T cells) ($r= 0.37$, $p=0.16$) T cells. (Figure 4.16A, 4.16B and 4.16C). In contrast no correlations were found between these cell types in the PBMC of JIA patients (Figure 4.16D, 4.16E and 4.16F).

Given that our group has previously demonstrated an inverse correlation between Th17 (CD4+ IL-17+) cells and Treg within SFMC, potential relationships between ILC3 and Treg in SFMC were next explored. Significance was not reached when ILC3 subpopulations were analysed against the proportion of CD4+ Treg in the PBMC or SFMC of JIA patients, however some trends were evident in SFMC. Thus a negative trend was seen between NCR-ILC3 as a proportion of total live cells and Foxp3+CD4+Treg as % of all CD4+ T cells at the inflamed site ($r=-0.379$, $p=0.20$) (Figure 4.17A). Conversely, a positive trend was seen between NCR+ILC3 as a proportion of total live cells and Foxp3+CD4+ Treg as % of all CD4+ T cells at the inflamed site ($r=0.495$, $p=0.08$) (Figure 4.17B). Although similar trends were seen when comparing these cell populations in PBMC, no significant associations were found between NCR- or NCR+ILC3 and Treg in the PBMC of JIA patients (Figure 4.17C and 4.17D).

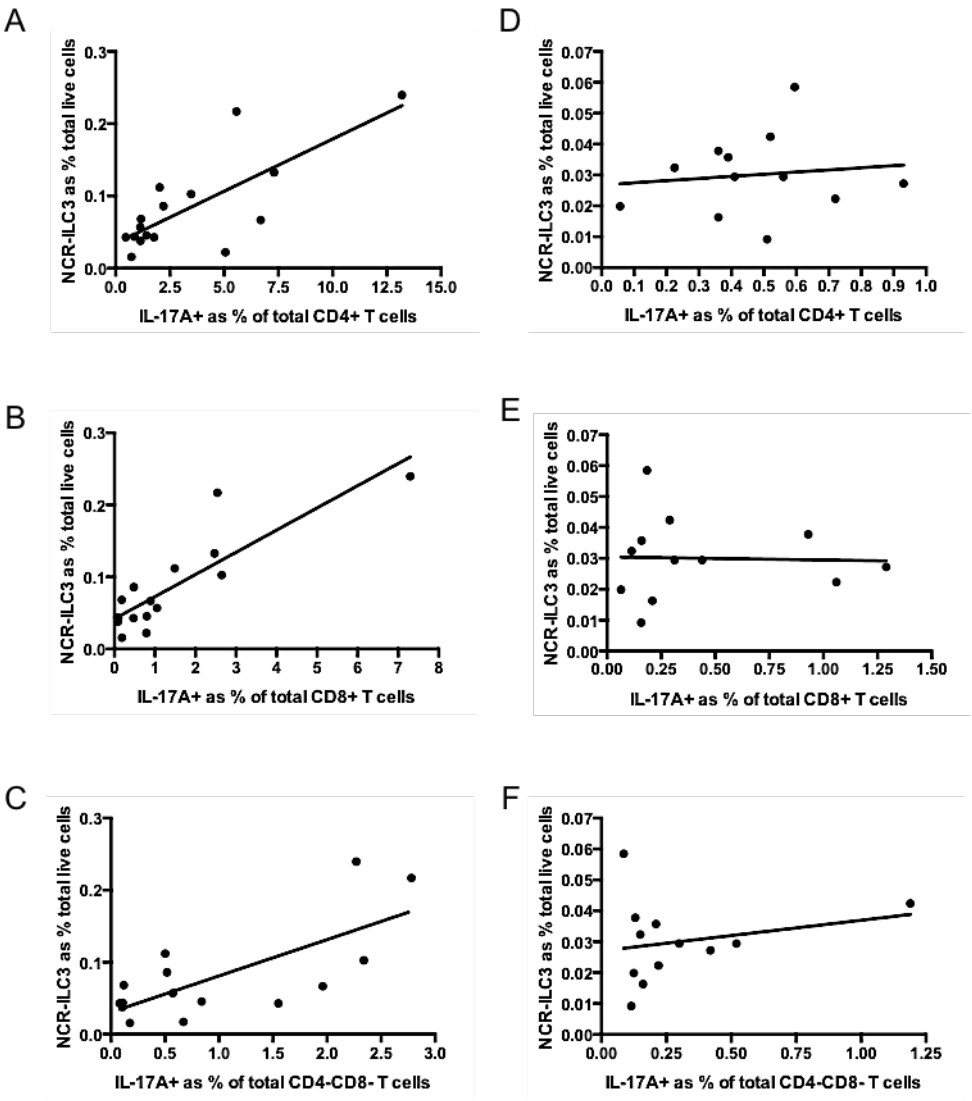


Figure 4.16 IL-17-producing ILC3 correlate with IL-17-producing CD4+ and CD8+ T cells within SFMC.

(A-C) Correlations in the SFMC of JIA patients between (A) NCR-ILC3 and IL-17+CD4+ T cells (n=18, r=0.49, p=0.042); (B) NCR-ILC3 and IL-17+CD8+ T cells (n=18, r=0. 59, p=0.011); (C) NCR-ILC3 and IL-17+CD4-CD8- T cells (n=18, r= 0.37, p=0.16) and (D-F) Correlations in the PBMC of JIA patients between (D) NCR-ILC3 and IL-17+CD4+ T cells (n=12, r=0.108, p=0.735); (E) NCR-ILC3 and IL-17+CD8+ T cells (n=12, r=0.102, p=0.754); (F) NCR-ILC3 and IL-17+CD4-CD8- T cells (n=12, r= 0.105, p=0.744). Correlation analysis by Spearman correlation

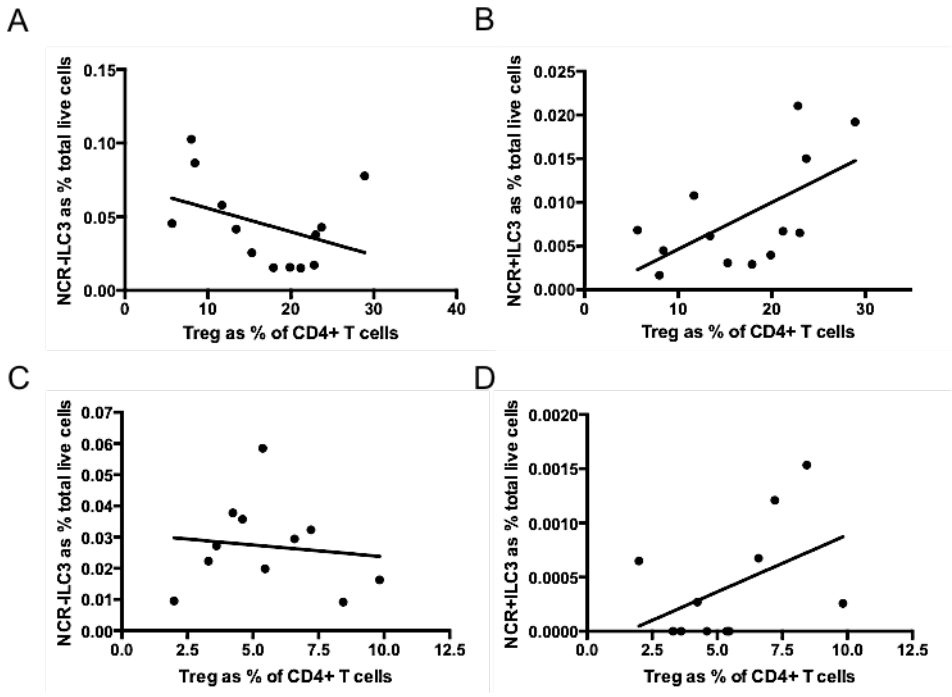


Figure 4.17 IL-22-producing ILC3 correlate with regulatory T cells within SFMC.

Correlations in the SFMC of JIA patients between (A) NCR-ILC3 and FOXP3+CD4+T cells (n=13, r=-0.379, p=0.20); (B) NCR+ILC3 and FOXP3+CD4+T cells (n=13, r=0.495, p=0.08) and correlations in the PBMC of JIA patients between (C) NCR-ILC3 and FOXP3+CD4+T cells (n=11, r=-0.146, p=0.673); (D) NCR+ILC3 and FOXP3+CD4+T cells (n=11, r=0.448, p=0.17) Correlation analysis by Spearman correlation.

4.3 Discussion

This chapter presented novel data characterizing ILC populations at the inflamed site in JIA, by analysis of SFMC and comparison to PBMC of both JIA patients and controls, and demonstrated the approaches taken to investigate their functionality and associations with disease pathogenesis.

ILC are a newly described population of lymphoid cells, which have been implicated in the pathogenesis of numerous inflammatory conditions via the production of influential cytokines (Spits and Cupedo, 2012). It is known that Th17 are important mediators of disease in JIA, and the significance of this population in ERA JIA was further illustrated in Chapter 3 of this thesis (Nistala et al., 2008a, Nistala and Wedderburn, 2009). Given the evidence for involvement of T cells in disease pathogenesis in JIA, the parallels that exist between T cells and ILC suggest that ILC may be an important new focus for investigation in relation to JIA (Nistala et al., 2008a, Mjosberg and Spits, 2016). The significance of the IL-17 and IL-23 axis is acknowledged in numerous autoimmune and inflammatory diseases, and new biological therapies targeting this pathway, such as Secukinumab which blocks IL-17 and Guselkumab and Ustekinumab which block IL-23 are now gaining momentum for proposed trials in JIA after proving useful for the treatment of adult ankylosing spondylitis, psoriasis and psoriatic arthritis (Chiricozzi and Krueger, 2013, McInnes et al., 2014, Poddubnyy et al., 2014, Sofen et al., 2014, Baeten et al., 2015). When considering a new biologic as a possible novel treatment, it is important to consider all the potential targets it may have. Similarly to Th17 cells, ILC3 populations are reliant on IL-1β and IL-23 for their development as well as their subsequent activation and have been shown be potent producers of IL-17 (Cupedo et al., 2009, Cella et al., 2010, Walker et al., 2013). It was therefore hypothesized that a significant enrichment of these ILC3 would be present at the inflamed site and that their proportion would be associated with a more severe disease status. ILC3 represent one population, which would likely be significantly affected by treatment with new biologics that target IL-17 or IL-23.

Unlike other immune cell types, no definitive protein markers have been identified which are exclusive to or specific for ILC. For this reason effective strategies for their exclusive identification have been difficult to develop and until recently there has been little consensus on the most appropriate approach for their analysis. In order to accurately characterize ILC at the inflamed site in JIA, it was imperative to validate an effective strategy for their identification. It is broadly accepted that cells defined as ILC are negative for markers associated with previously defined lineages (T cells, B cells, NK cells, monocytes, DC and granulocytes), and that ILC rely on IL-7 for their development and so express high levels of the IL-7Rα chain (CD127) (Neill et al., 2010, Wong et al., 2012). Additionally, ILC are defined as having lymphoid morphology, which is associated with characteristically low forward and side scatter properties when analysed by flow cytometry (Hazenberg and Spits, 2014a).

Cells from the synovial fluid of JIA patients are highly activated and therefore lymphocytes typically have a higher forward and side scatter profile than seen in PBMC. Therefore, larger cells should, where possible, be included in analyses of SFMC in order to avoid exclusion of activated ILC. Identification of ILC using lineage markers and CD127 expression alone appears to be insufficient in the inflamed site due to the emergence of a large lineage^{low}CD127+ population of cells in the synovial fluid with unexpectedly high scatter properties, which suggested a further cell population had been identified which were lineage^{low}CD127+. Due to the increased size of cells in the SFMC, it is possible that this lineage^{low} population were in fact ILC with high auto fluorescence. It is also possible that some protein markers were relatively down regulated on the surface of some lineage positive cells due to their chronic activation within the joint leading to the appearance of a lineage^{low} population of cells. To address this issue, additional markers were added to the ILC selection panel. NK cell receptor CD161 was suggested by a group of experts as a characteristic marker of CD127+ ILC(Spits et al., 2013). CD161 is a known NK- cell marker also expressed on subpopulations of CD8 and CD4 T cells but is now also accepted to be expressed on ILC(Cosmi et al., 2008, Maggi et al., 2010, Mjosberg et al., 2011b, Pesenacker et al., 2013). The inclusion of the analysis of CD161 in this study allowed for identification of an ILC population in blood and synovial fluid of

patients irrespective of cell size. In order to maintain consistency between samples, and to ensure that the same ILC population was analysed between blood and SFMC, expression of CD161 was subsequently considered an essential criterion for ILC classification throughout this project.

No significant difference was seen between the total numbers of ILC in the joint compared the blood of JIA patients. It is important to consider that ILC are generally considered to be tissue resident cells(Hazenberg and Spits, 2014a). Due to ethical restrictions meaning that the majority of children undergoing joint fluid aspiration do not have synovial biopsy material sampled, it was not possible to analyse ILC within the synovial tissue of JIA patients in this study, but such analysis would surely be interesting. Additionally, despite stringent optimization, data from culture assays performed indicate that identification of synovial ILC may be significantly more complicated in the synovia than previously appreciated. Preliminary results after culture of aHC ILC in activating cytokines showed that activation of ILC may lead to down regulation of surface protein levels CD127 and CD161, two markers essential for ILC identification. Unlike peripheral blood ILC, synovial ILC are likely to be highly activated. It is therefore reasonable to suggest that a proportion of activated ILC in the joint may have down regulated these influential markers and therefore may not be detected via current analyses. It is therefore possible that the ILC proportions detected in this study within SFMC samples could represent an underestimation of the true ILC population.

This study has shown significantly altered ILC signatures between the mononuclear cell populations from blood and joints of JIA patients as determined by surface receptor, cytokine expression and transcription factor analysis. In agreement with previously published data, the ILC analysis performed here shows ILC2 to be the predominant ILC population in peripheral blood of healthy controls (adults and children) as well as JIA patients (Mjosberg et al., 2011b). A significant ablation of ILC2 as a proportion of total ILC detected was seen in the SFMC of JIA patients in accordance with previously published data in other inflammatory settings(Leijten et al., 2015). This raises the question as to whether ILC subtypes are selectively recruited to the joint, or whether the

inflamed synovial environment leads to downregulation of the CRTH2 marker, and ILC plasticity.

NCR+ILC3 were almost completely absent from peripheral blood samples, while similarly to results shown in psoriatic skin and psoriatic arthritis, a significant enrichment of NCR+ILC3 was detected at in SFMC samples of JIA patients(Teunissen et al., 2014a, Leijten et al., 2015). Furthermore, these cells showed an association with less severe disease as determined by physician's global disease visual analogue score, (VAS). Although this is a subjective assessment of disease severity, it is intriguing in the context of these IL-22 producing cells. The link with improved physicians VAS, without association with ESR or active joint count, suggests that these cells are related to the patient feeling and presenting better to the clinician even though on a biochemical level they may still have significant inflammation (measured by ESR). It is interesting that there was no clear association seen between NCR+ILC3 and JADAS3 scores. The JADAS3 includes the physician's VAS score as well as the parent global score of disease and active joint count. This may be due to the low power of this study, in part due to significant missing data. The association seen between NCR+ILC3 and regulatory T cells in the synovial fluid further supported the concept that these cells may be having a 'protective' influence. Although IL-22 is often considered to be an inflammatory cytokine, it is also implicated in homeostasis and repair mechanisms in the skin and other organs. In the skin, IL-22 to has been shown to promote wound healing through interaction with fibroblasts (McGee et al., 2013). NCR+ILC3 have also been shown to play a significant role in maintaining barrier integrity in the gut (Geremia et al., 2011). It is possible that NCR+ILC3 mediate reparatory actions via IL-22 production and interaction with synovial fibroblasts.

IL-17 producing T cells have been identified in JIA and are implicated in disease pathogenesis (Nistala et al., 2008a). In parallel to IL-17 producing T cell subtypes, NCR-ILC3 were significantly enriched in the joints of JIA patients. These finding supports the hypothesis that the IL-17 signature extends beyond the T cell compartment in some patients and is further reinforced by the correlations that were found between these different cell types. The strong

associations shown between NCR-ILC3 and measures of disease severity implicate this population in disease pathogenesis. It is clear that this ILC subset may also be targeted when considering treatments, which are designed to ablate IL-17 in these patients. Animal models of arthritis would be useful tools to further investigate the true extent of ILC3 involvement in disease progression and protection. Unlike in the T cell data presented in Chapter 3, NCR-ILC3 were not significantly enriched in ERA JIA patients compared to other JIA subtypes. This may be due to an insufficient number of patient samples being analysed for other JIA subtypes.

As ILC are considered to be innate immune cells it would be reasonable to suggest that the role of ILC could be relatively restricted to the initiation and early stages of disease. However, while they are important for the induction of acute inflammation in response to pathogen, considerable evidence suggests that they also play pivotal roles in resolution from inflammation as well as repair (Dudakov et al., 2012, Gladiator et al., 2013). Preliminary longitudinal analysis of ILC subtypes in synovial fluid over time shows that relative proportions can vary over time with disease severity and higher proportions of NCR-ILC3 are found at time points when patients were experiencing more severe symptoms.

Taken together, the results shown in this chapter demonstrate an altered ILC phenotype in the SFMC compared to blood of JIA patients with a significant enrichment of two populations of group 3 ILC (NCR-ILC3 ad NCR+ILC3), able to produce IL-17 and IL-22 at the inflamed site. Synovial NCR-ILC3, known to be potent producers of inflammatory IL-17 are associated with more severe disease, suggesting a role for these cells in disease pathogenesis. Additionally, NCR-ILC3 are correlated with IL-17-producing T cell subsets, supporting the initial hypothesis that the IL-17 signature extends beyond the T cell compartment in these patients. In parallel a significant enrichment of IL-22 producing NCR+ILC3 was detected in the joint. Conversely to their IL-17-producing counterparts, these NCR+ILC3 were found to be associated with less severe disease when the patients are assessed holistically via the physicians VAS. Further supporting the suggestion that these cells may play a protective role, NCR+ILC3 were shown to correlate with regulatory T cells at in the

synovial fluid. The following chapter will focus on elucidating a rationale behind the altered ILC phenotype observed at the inflamed site in JIA patients.

5 : Investigation of Innate Lymphoid Cell Migration and Plasticity

5.1 Introduction

The data presented in Chapter 4 demonstrated an enrichment of IL-17 and IL-22 producing ILC3 and an ablation of ILC2 within the mononuclear cell compartment at the inflamed site in children with arthritis, compared to the peripheral blood mononuclear cell population. The mechanisms underlying how this signature is achieved in the joints of JIA patients is currently unclear.

There is relatively little known about ILC recruitment to inflamed sites; a recent study has shown that chemokine receptor expression on ILC largely mirrors their respective T cell subpopulation counterparts (Roan et al., 2016b). Significant presence of chemokines such as CCL2, CCL5, CCL20 and CXCL10 and an enrichment of T cells expressing their corresponding receptors, CCR4, CCR5, CCR6 and CXCR3, has been demonstrated in the inflamed synovial fluid of patients with JIA (Wedderburn et al., 2000b, Pharoah et al., 2006, Nistala et al., 2008b, Issekutz et al., 2011). To date, no studies have reported on ILC migration and their presence in the inflamed site in JIA. One possible explanation for the altered ILC phenotype in the joint (compared to PBMC) could be due to the selective recruitment of ILC via the interaction of chemokine receptors on ILC with their ligands at the inflamed site.

There is now significant evidence supporting plasticity between ILC subpopulations (Artis and Spits, 2015). Thus it is clear that under the influence of a specific cytokine/chemokine milieu, ILC may 'switch' their phenotype/functional characteristics. Several groups have explored the effects of cytokines in promoting the transition of ILC between subpopulations. There is clear plasticity between ILC1 and ILC3 subpopulations. Similarly to T cells, under the influence of IL-12, ILC3 are shown to skew towards an ILC1 phenotype (Nistala et al., 2010a, Bernink et al., 2013a, Bernink et al., 2015). Conversely, ILC1 can be driven towards ILC3 by culture with IL-1β and IL-23 (Bernink et al., 2015). There is even some recent indication that inflammatory cytokines can induce ILC2 switching to other ILC subpopulations (Silver et al., 2016a). As plasticity has previously been demonstrated in T cell subpopulations

in the joint in JIA (Nistala et al., 2010b), it is reasonable to suggest that cytokines at the inflamed site in JIA may also drive changes in ILC phenotype, potentially switching towards ILC1 and ILC3 populations.

The aim of this Chapter was to investigate mechanism(s) that lead to the altered relative proportions of ILC subpopulations at the inflamed site compared to the peripheral blood compartment. I hypothesized that selective migration of specific subpopulation of ILC, in response to specific chemokines contributes to ILC recruitment to the inflamed site. I additionally hypothesized that cytokines at the inflamed site would induce ILC phenotype switching towards the ILC1/ILC3 subpopulations.

Experimental objectives:

1. To investigate the chemokines in the joint and the expression of their respective receptors on synovial ILC populations.
2. To investigate cytokines at the inflamed site and their effects on ILC plasticity.
3. To investigate cells within the SFMC which may contribute to the cytokine milieu and therefore influence the ILC phenotype.

5.2 Results

5.2.1 Differential chemokine receptor expression on synovial ILC compared to healthy ILC

Chapter 4 demonstrated an altered ILC phenotype in the synovial fluid mononuclear cells from joints of JIA patients compared to their peripheral blood: however the mechanisms by which this distinct population profile is achieved are still unclear. In order to test the hypothesis that ILC are selectively recruited to the inflamed site due to specific chemokine gradients, the concentration of specific chemokines and the expression of their respective receptors on synovial ILC was assessed, by multiplex chemokine analysis and flow cytometry, respectively.

Multiplex chemokine analysis (Luminex platform) was carried out on the JIA patient cohort in collaboration with Dr W. de Jager, University of Utrecht (de Jager et al., 2007). 16 paired JIA SF and blood serum samples, collected at time of active clinical disease when patient attend clinic for joint injection, were analysed to determine the relative concentrations of CCL2, CCL3, CCL4, CCL8, CCL20, CCL25, CXCL6, CXCL8, CXCL9, CXCL10 and CXCL11 quantification (Figure 5.1). The dynamic range of the Luminex assay is ~5pg/ml-10,000pg/ml. For concentrations above this range, values were generated by extrapolation of the standard curve. Concentrations below the lower limit of detection were allocated a concentration of 0pg/ml, however the true value is likely to be above zero.

The synovial fluid concentrations of the detected chemokines were highly variable between clinical samples. Significantly higher concentrations of CCL2, CCL20, CXCL8, CXCL9 and CXCL10 were observed in the SF (samples collected and stored by same protocol as serum), compared to serum (Figure 5.1 A-E, Table 5.1). No differences were seen in the concentrations of CCL3, CCL4, CCL8, CCL25, CXCL6 or CXCL11 between serum and SF (Figure 5.1F-K, Table 5.1).

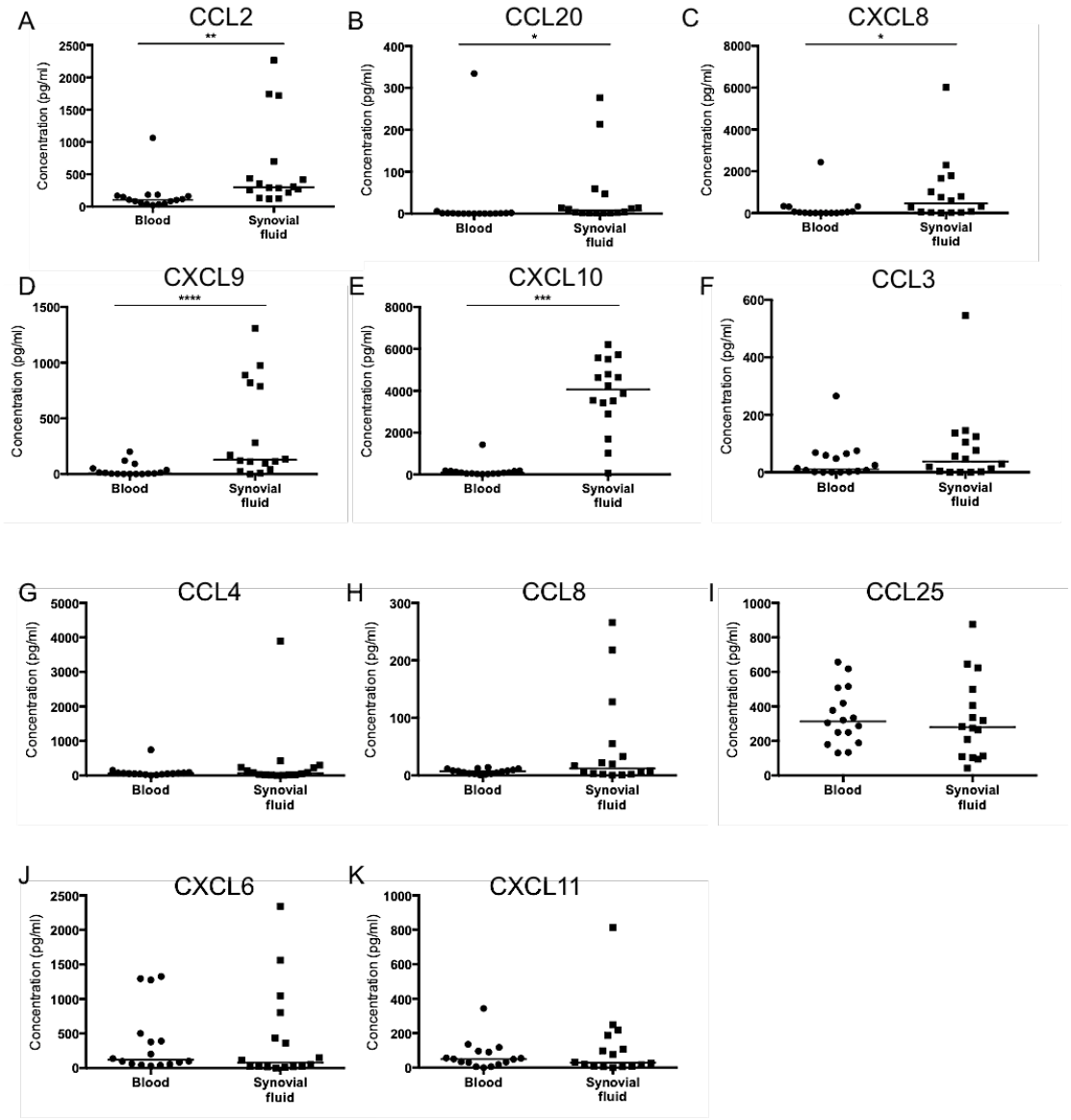


Figure 5.1 Multiplex chemokine analysis of synovial fluid and peripheral blood serum from JIA patients.

Paired JIA SF and peripheral blood serum samples (n=16), collected at time of active clinical disease when patients attend for joint injection, were analysed for specific chemokine concentrations using the Luminex platform. Summary plots showing concentrations of (A) CCL2; (B) CCL20; (C) CXCL8; (D) CXCL9; (E) CXCL10; (F) CCL3; (G) CCL4; (H) CCL8; (I) CCL25; (J) CXCL6 and (K) CXCL11. Statistical analysis carried out by paired T test. Bars represent median values. * $p<0.05$ ** $p<0.01$ *** $p<0.001$ **** $p<0.0001$.

	Serum Median (IQR)	SF Median (IQR)
CCL2	102.6 (46.88-165.4)	299.2 (228.0-633.8)
CCL3	10.34 (1.19-63.19)	37.8 (2.45-119.0)
CCL4	60.84 (39.13-74.85)	69.21 (14.41-231.2)
CCL8	7.0 (3.38-10.86)	11.76 (2.76-49.64)
CCL20	0.54 (0-1.21)	7.22 (1.79-38.65)
CCL25	312.5 (203.9-485.9)	278.6 (110.3-476.2)
CXCL6	118.4 (56.19-437.8)	80.49 (21.13-711.4)
CXCL8	30.14 (0.09-244)	454.3 (38.81-1502)
CXCL9	7.76 (0.7-46.78)	128.3 (54.93-812.4)
CXCL10	85.55 (45.59-162.7)	4056 (3025-5331)
CXCL11	49.64 (21.75-95.04)	29.13 (8.43-166.8)

Table 5.1 Concentrations of cytokines in serum and SF from JIA patients.

Based on the results from the multiplex chemokine analysis, cell surface chemokine receptor expression was assessed on peripheral blood and synovial ILC by flow cytometry in order to gain evidence for selective ILC recruitment to the inflamed site.

***CXCR3+ ILC*:** CXCR3 is the receptor for chemokines CXCL9, CXCL10 and CXCL11 (Groom and Luster, 2011). Both CXCL9 and CXCL10 were shown to have significantly elevated concentrations in the SF compared to serum from JIA patients (Figure 5.1D and 5.1E). After gating for ILC (CD45+lineage-CD127+CD161+), as shown in Chapter 4, the expression of CXCR3 was analysed according to % CXCR3+ ILC (Figure 5.2A) and MFI of CXCR3 (Figure 5.2B) on the total ILC population and subsequently on the individual ILC subpopulations. A significant increase in the expression of CXCR3 on total ILC was identified, when analysed either by %CXCR3+ cells within the ILC

population, (Figure 5.2C) or by MFI on the total population (Figure 5.2D), ($p=0.036$ and $p=0.0006$ respectively). Interestingly, 3 of the 5 SFMC samples tested showed very high CXCR3 expression on total ILC (Figure 5.2C and 5.2D). When the ILC subpopulations (ILC1, ILC2, NCR-ILC3 and NCR+ILC3) were studied (Figure 5.2E and 5.2F), CXCR3 expression was found to be highest on the SFMC ILC1 population: this expression was significantly greater compared to paired PBMC ILC1 ($p<0.005$ for %CXCR3+ and MFI) (Figures 5.2G and 5.2H). A trend towards high expression of CXCR3 was also observed on NCR-ILC3 synovial population but this was not significantly different from the expression on blood NCR-ILC3.

***CCR4+ ILC*:** CCR4+ cells migrate towards CCL2, CCL4, CCL17 and CCL22 (Yoshie and Matsushima, 2015). Statistically significant CCL2 protein levels were recorded at the inflamed site compared to serum levels (Figure 5.1A). Next, the cell surface CCR4 expression was investigated. Percentage CCR4+ ILC and MFI of CCR4 are shown in Figure 5.3A and 5.3B respectively. Surprisingly, CCR4 expression on total ILC was reduced on synovial ILC compared to paired PBMC ILC by both %CCR4+ cells ($p<0.0001$) and MFI ($p<0.0001$)(Figures 5.3C and 5.3D). This could be in part due to the relative paucity of ILC2 cells within SFMC compartment (Chapter 4). When ILC subpopulations were analysed (Figure 5.3E and 5.3F), ILC2 showed significantly higher CCR4 cell surface expression compared to other ILC subpopulations ($p=0.008$). No significant difference was detected in CCR4 expression on ILC2 SFMC and PBMC compartments. However CCR4 expression was significantly reduced on synovial ILC1 ($p=0.04$) and appeared to show a trend towards reduced expression on NCR-ILC3 populations in SFMC compared to PBMC of JIA patients (Figures 5.3G and 5.3H).

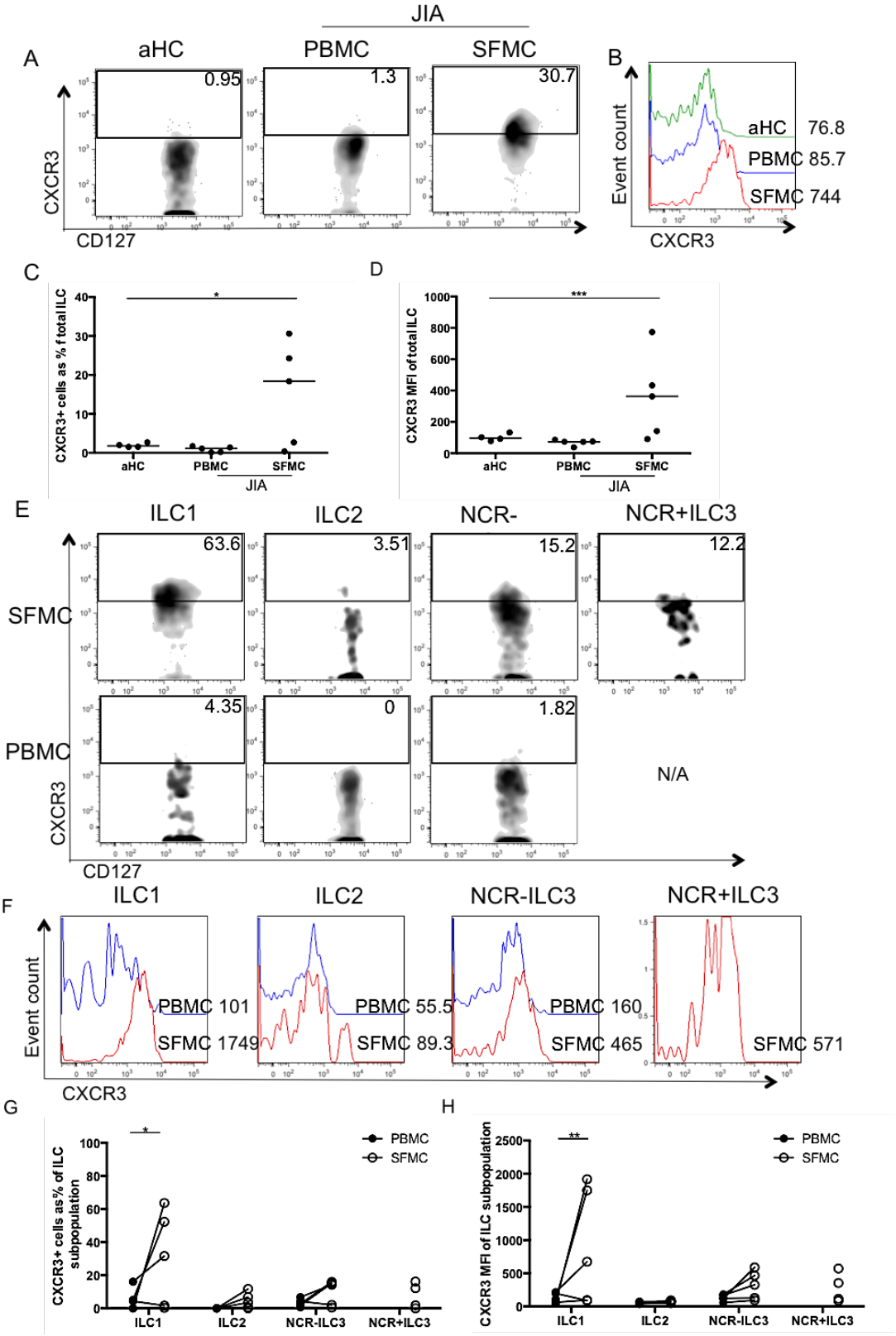

JIA
A
aHC
PBMC
SFMC
0.95
1.3
30.7
CXCR3
CD127
B
Event count
aHC 76.8
PBMC 85.7
SFMC 744
CXCR3
C
CXCR3+ cells as % f total ILC
aHC
PBMC
SFMC
JIA
D
CXCR3 MFI of total ILC
aHC
PBMC
SFMC
JIA
E
ILC1
ILC2
NCR-
NCR+ILC3
SFMC
PBMC
63.6
3.51
15.2
12.2
4.35
0
1.82
N/A
CXCR3
CD127
F
ILC1
ILC2
NCR-ILC3
NCR+ILC3
Event count
PBMC 101
SFMC 1749
PBMC 55.5
SFMC 89.3
PBMC 160
SFMC 465
SFMC 571
CXCR3
G
CXCR3+ cells as% of ILC subpopulation
PBMC
SFMC
ILC1
ILC2
NCR-ILC3
NCR+ILC3
H
CXCR3 MFI of ILC subpopulation
PBMC
SFMC
ILC1
ILC2
NCR-ILC3
NCR+ILC3

Figure 5.2 CXCR3 is expressed more highly on ILC1 relative to other ILC populations

Expression of chemokine receptor CXCR3 measured on total ILC from aHC PBMC (n=4) and PBMC (n=5) and SFMC (n=5) from JIA patients. (A) Representative plots showing the gating strategy for the identification of CXCR3 positive cells as a % of total ILC (CD45+lineage-CD127+CD161+) (B) Representative histograms showing the protein expression of CXCR3 by MFI on total ILC from one aHC PBMC, JIA PBMC and SFMC sample (C) Summary plot showing CXCR3 positive cells as % of total ILC. (D) Summary plot showing protein expression of CXCR3 by MFI on total ILC. (E) Representative plots showing the strategy used for the identification of CXCR3 positive cells as a % of each ILC subpopulation (ILC1, ILC2, NCR-ILC3 and NCR+ILC3) from paired PBMC and SFMC from JIA patients. (F) Representative histograms showing the protein expression of CXCR3 by MFI on each ILC subpopulation (ILC1, ILC2, NCR-ILC3 and NCR+ILC3) from paired PBMC and SFMC from JIA patients. (G) Summary plot showing CXCR3 positive cells as % of ILC subpopulations. (H) Summary plot showing protein expression of CXCR3 by MFI on ILC subpopulations. Statistical analysis carried out by Kruskal – Wallis or by paired T test. Bars represent median values * $p<0.05$ ** $p<0.01$

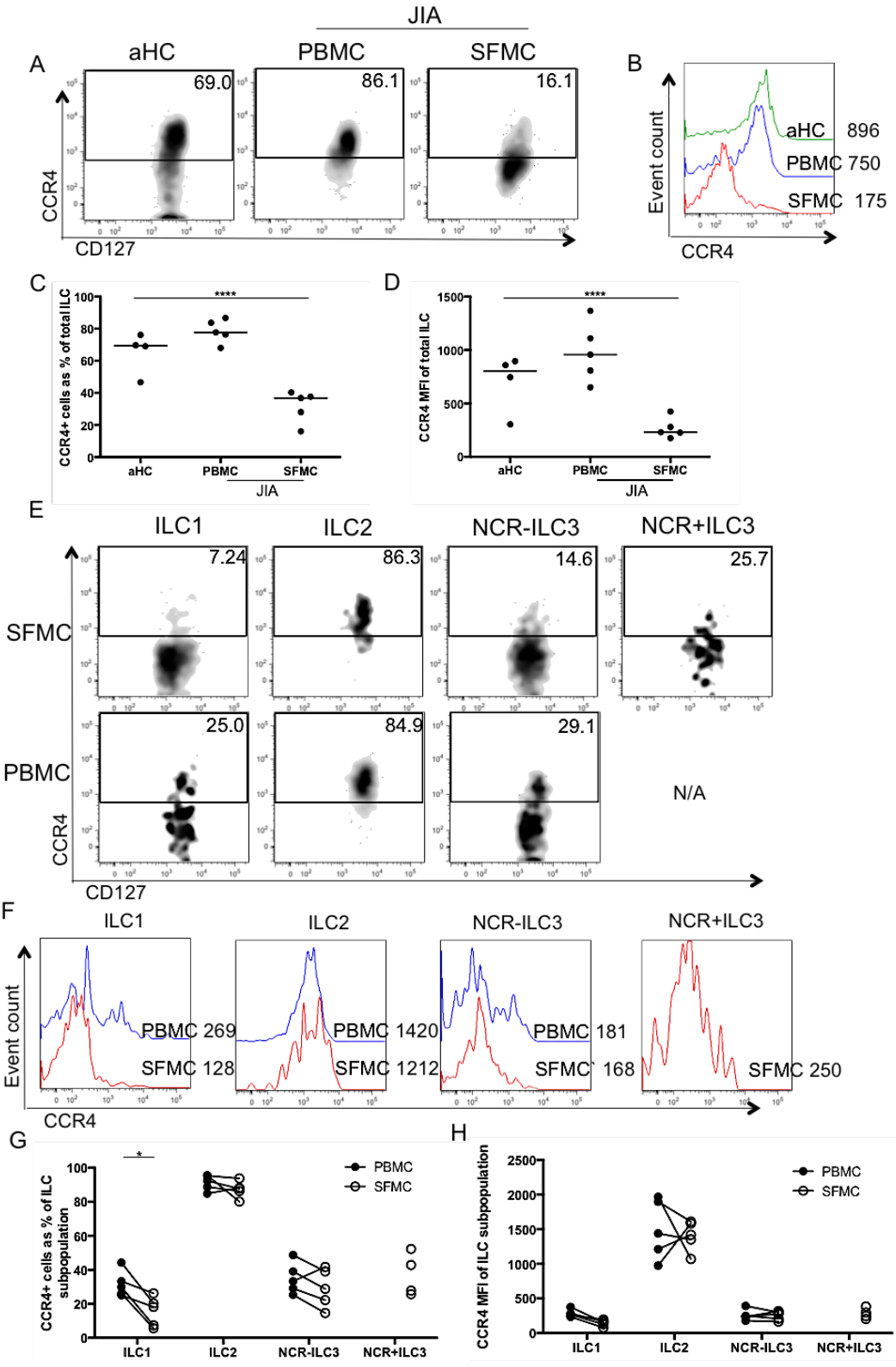

JIA
A
aHC
PBMC
SFMC
69.0
86.1
16.1
CCR4
CD127
B
Event count
aHC 896
PBMC 750
SFMC 175
CCR4
C
CCR4+ cells as % of total ILC
****
aHC
PBMC
SFMC
JIA
D
CCR4 MFI of total ILC
****
aHC
PBMC
SFMC
JIA
E
ILC1
ILC2
NCR-ILC3
NCR+ILC3
SFMC
7.24
86.3
14.6
25.7
PBMC
25.0
84.9
29.1
N/A
CCR4
CD127
F
ILC1
ILC2
NCR-ILC3
NCR+ILC3
Event count
PBMC 269
SFMC 128
PBMC 1420
SFMC 1212
PBMC 181
SFMC 168
SFMC 250
CCR4
G
*
PBMC
SFMC
CCR4+ cells as % of ILC subpopulation
ILC1
ILC2
NCR-ILC3
NCR+ILC3
H
PBMC
SFMC
CCR4 MFI of ILC subpopulation
ILC1
ILC2
NCR-ILC3
NCR+ILC3

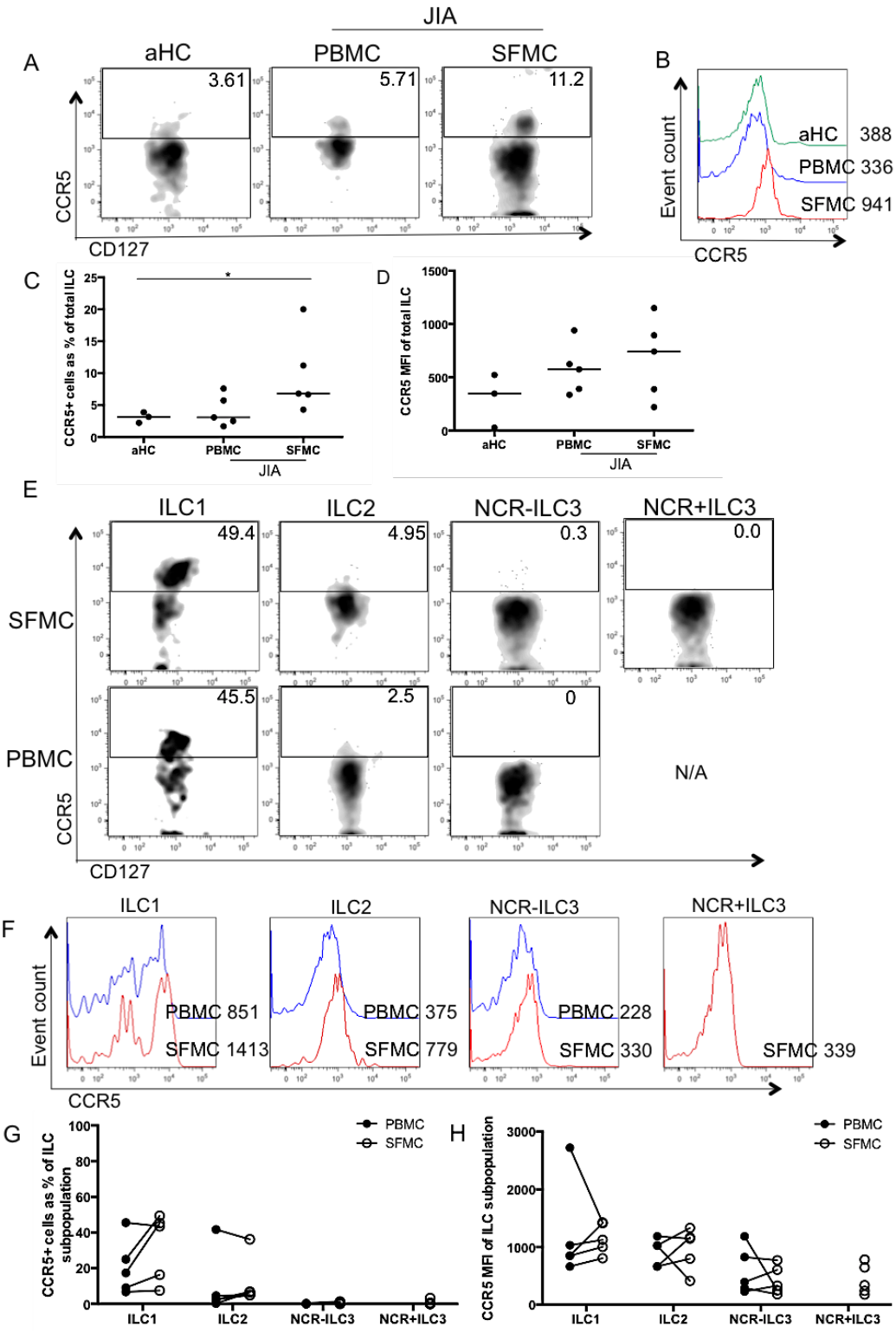
Figure 5.3 CCR4 is highly expressed on ILC2 relative to other ILC populations

Expression of chemokine receptor CCR4 measured on total ILC from aHC PBMC (n=4) and PBMC (n=5) and SFMC (n=5) from JIA patients. (A) Representative plots showing the gating strategy for the identification of CCR4 positive cells as a % of total ILC (CD45+lineage-CD127+CD161+) (B) Representative histograms showing the protein expression of CCR4 by MFI on total ILC from one aHC PBMC, JIA PBMC and SFMC sample (C) Summary plot showing CCR4 positive cells as % total ILC. (D) Summary plot showing protein expression of CCR4 by MFI on total ILC. (E) Representative plots showing the strategy used for the identification of CCR4 positive cells as a % of each ILC subpopulation (ILC1, ILC2, NCR-ILC3 and NCR+ILC3) from paired PBMC and SFMC from JIA patients. (F) Representative histograms showing the protein expression of CCR4 by MFI on each ILC subpopulation (ILC1, ILC2, NCR-ILC3 and NCR+ILC3) from paired PBMC and SFMC from JIA patients. (G) Summary plot showing CCR4 positive cells as % of ILC subpopulations. (H) Summary plot showing protein expression of CCR4 by MFI on ILC subpopulations. Statistical analysis carried out by Kruskal – Wallis or by paired T test. Bars represent median values * $p<0.05$ ****$p<0.0001$

***CCR5+ ILC*:** Despite low concentrations of CCL3 and CCL4 in the serum and SF samples, the expression of CCR5 on ILC was also analysed given that it is known to interact with RANTES (CCL5), which has been previously shown to be abundant at the inflamed site in JIA (Figures 5.2-5.5) (Pharoah et al., 2006). Within the whole ILC population, the % of CCR5+ ILC was significantly higher in the SFMC compared to PBMC from JIA patients (p=0.049) (Figure 5.4A and 5.4C). No significant difference was seen in MFI of CCR5 between PBMC and SFMC (Figure 5.4B and 5.4D), although a trend towards a larger CCR5+ ILC1 population was seen in the SFMC (p=0.102) (Figure 5.4E and 5.4G). No difference was seen in the MFI of CCR5 expression on total ILC between SFMC and PBMC, or between ILC subpopulations, which may be due to high variability and underpowered analyses (Figures 5.4B, 5.4F and 5.4H).

***CCR6+ ILC*:** CCR6 the receptor for CCL20, which this study and others have demonstrated to be elevated at the inflamed site in several types of inflammatory arthritis. The expression of CCR6 was analysed according to % CCR6+ ILC (Figure 5.5A) and MFI of CCR6 (Figure 5.5B) on ILC within both SFMC and PBMC. Surprisingly, no significant difference in CCR6 expression on total synovial ILC was observed according to either %CCR6+ cells (Figure 5.5C) or by MFI (Figure 5.5D). Additionally, no difference was seen in CCR6 expression between the ILC subpopulations. However, significantly higher CCR6 expression was detected on synovial ILC2 compared to paired PBMC ILC2 (p=0.049) (Figures 5.5E-H). It should be noted that in the synovial fluid a significant increase in CCL20 may have been driven by high levels in a minority of samples and there was considerable heterogeneity in sample concentrations (Figure 5.1). Therefore, a larger set for samples may need to be analysed to understand the heterogeneity of chemokine receptor expression in the ILC populations found in the JIA synovial fluid cells.

CXCL8 has been predominantly been described to be significant for the migration of neutrophils to the sites of inflammation and was therefore not examined further in this investigation (Russo et al., 2010).


JIA
A
aHC
PBMC
SFMC
3.61
5.71
11.2
CCR5
CD127
B
Event count
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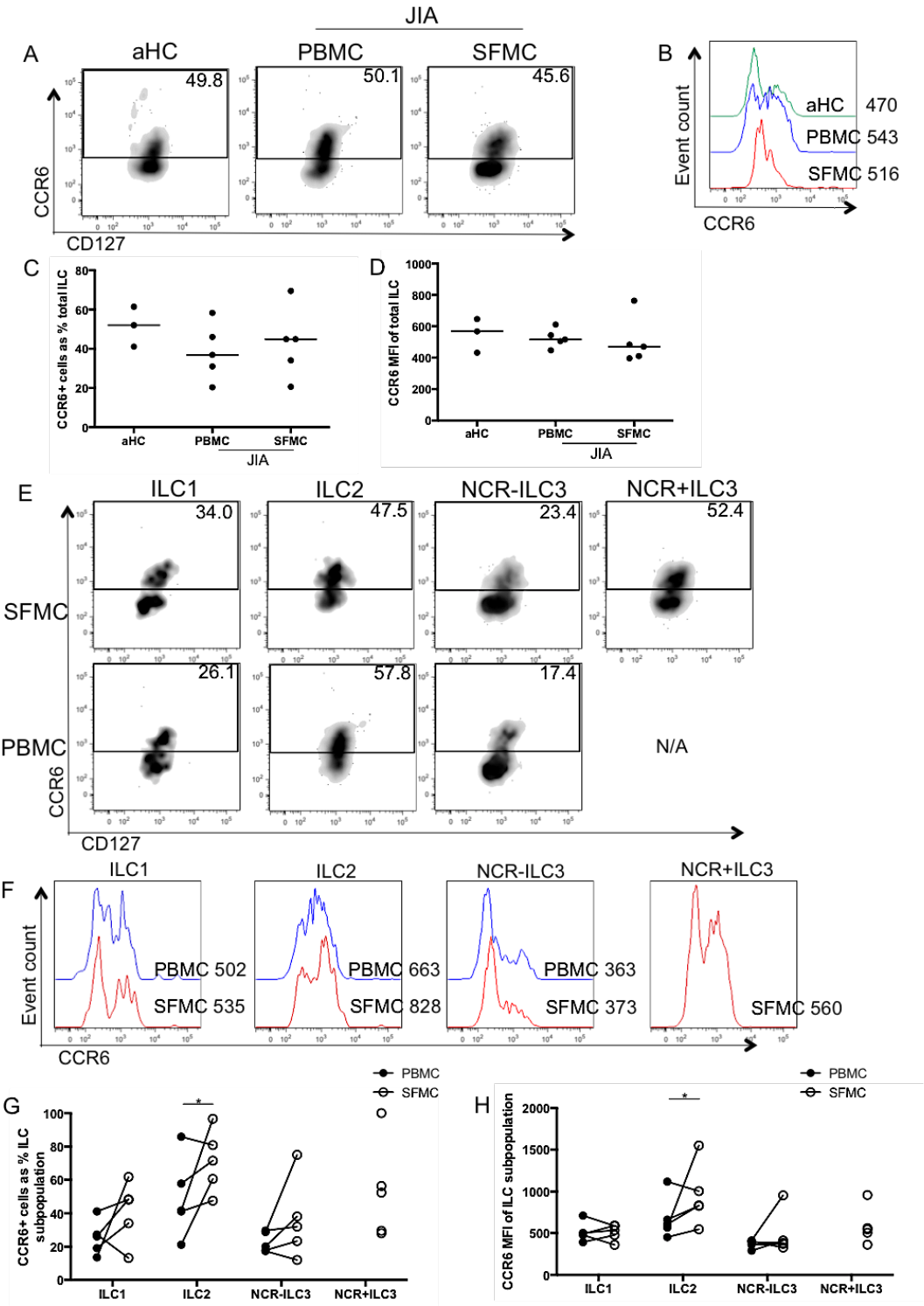
PBMC 336
SFMC 941
CCR5
C
CCR5+ cells as % of total ILC
aHC
PBMC
SFMC
JIA
D
CCR5 MFI of total ILC
aHC
PBMC
SFMC
JIA
E
ILC1
ILC2
NCR-ILC3
NCR+ILC3
SFMC
PBMC
49.4
4.95
0.3
0.0
45.5
2.5
0
N/A
CCR5
CD127
F
ILC1
ILC2
NCR-ILC3
NCR+ILC3
Event count
PBMC 851
SFMC 1413
PBMC 375
SFMC 779
PBMC 228
SFMC 330
SFMC 339
CCR5
G
PBMC
SFMC
CCR5+ cells as % of ILC subpopulation
ILC1
ILC2
NCR-ILC3
NCR+ILC3
H
PBMC
SFMC
CCR5 MFI of ILC subpopulation
ILC1
ILC2
NCR-ILC3
NCR+ILC3

Figure 5.4 CCR5 is expressed on ILC1 relative to other ILC populations

Expression of chemokine receptor CCR5 measured on total ILC from aHC PBMC (n=3) and PBMC (n=5) and SFMC (n=5) from JIA patients. (A) Representative plots showing the gating strategy for the identification of CCR5 positive cells as a % of total ILC (CD45+lineage-CD127+CD161+) (B) Representative histogram showing the protein expression of CCR5 by MFI on total ILC from one aHC PBMC, JIA PBMC and SFMC sample (C) Summary plot showing CCR5 positive cells as % total ILC. (D) Summary plot showing protein expression of CCR5 by MFI on total ILC. (E) Representative plots showing the strategy used for the identification of CCR5 positive cells as a % of each ILC subpopulation (ILC1, ILC2, NCR-ILC3 and NCR+ILC3) from paired PBMC and SFMC from JIA patients. (F) Representative histograms showing the protein expression of CCR5 by MFI on each ILC subpopulation (ILC1, ILC2, NCR-ILC3 and NCR+ILC3) from paired PBMC and SFMC from JIA patients. (G) Summary plot showing CCR5 positive cells as % of ILC subpopulations. (H) Summary plot showing protein expression of CCR5 by MFI on ILC subpopulations. Statistical analysis carried out by Kruskal – Wallis or by paired T test. Bars represent median values * $p<0.05$


JIA
A
aHC
PBMC
SFMC
49.8
50.1
45.6
CCR6
CD127
B
Event count
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PBMC 543
SFMC 516
CCR6
C
CCR6+ cells as % total ILC
aHC
PBMC
SFMC
JIA
D
CCR6 MFI of total ILC
aHC
PBMC
SFMC
JIA
E
ILC1
ILC2
NCR-ILC3
NCR+ILC3
34.0
47.5
23.4
52.4
SFMC
26.1
57.8
17.4
PBMC
N/A
CCR6
CD127
F
ILC1
ILC2
NCR-ILC3
NCR+ILC3
Event count
PBMC 502
SFMC 535
PBMC 663
SFMC 828
PBMC 363
SFMC 373
SFMC 560
CCR6
G
PBMC
SFMC
CCR6+ cells as % ILC subpopulation
ILC1
ILC2
NCR-ILC3
NCR+ILC3
H
PBMC
SFMC
CCR6 MFI of ILC subpopulation
ILC1
ILC2
NCR-ILC3
NCR+ILC3

Figure 5.5 CCR6 is expressed on ILC2 from SFMC compared to ILC2 from paired PBMC

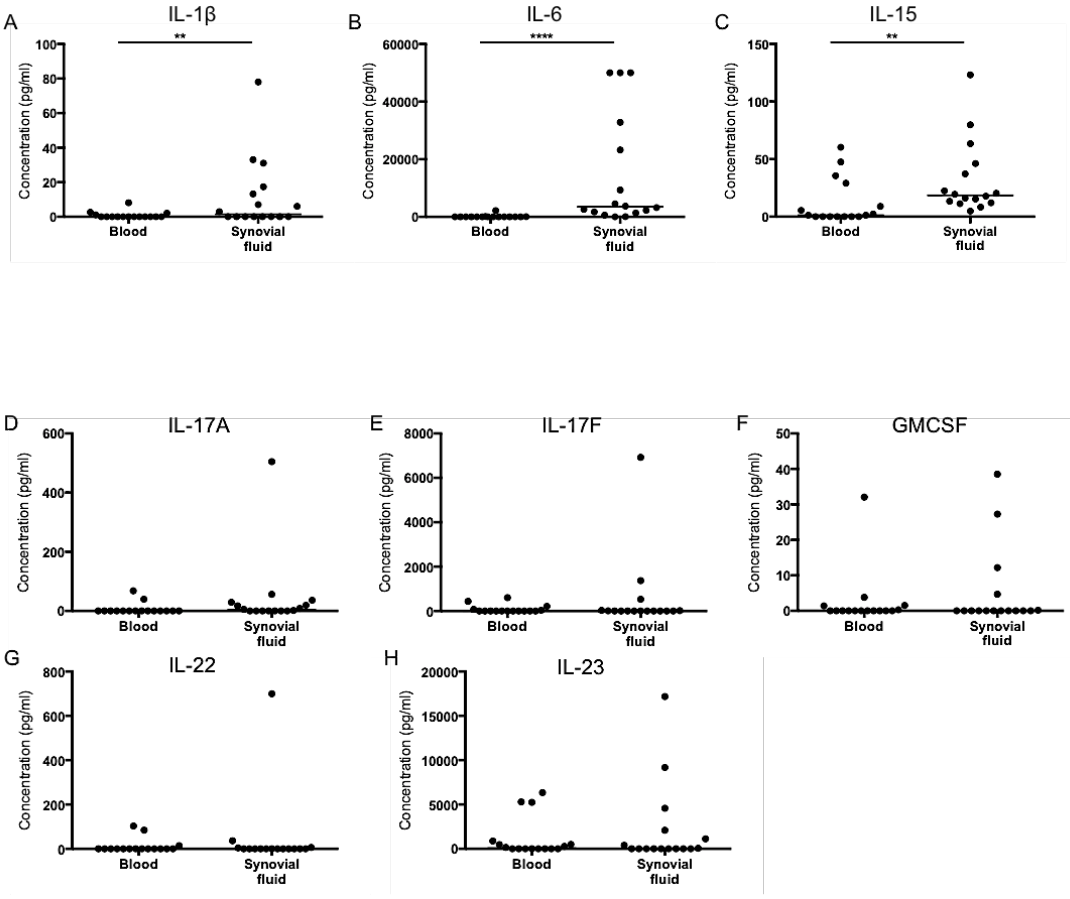
Expression of chemokine receptor CCR6 measured on total ILC from aHC PBMC (n=3) and PBMC (n=5) and SFMC (n=5) from JIA patients. (A) Representative plots showing the gating strategy for the identification of CCR6 positive cells as a % of total ILC (CD45+lineage-CD127+CD161+) (B) Representative histogram showing the protein expression of CCR6 by MFI on total ILC from one aHC PBMC, JIA PBMC and SFMC sample (C) Summary plot showing CCR6 positive cells as % total ILC. (D) Summary plot showing protein expression of CCR6 by MFI on total ILC. (E) Representative plots showing the strategy used for the identification of CCR6 positive cells as a % of each ILC subpopulation (ILC1, ILC2, NCR-ILC3 and NCR+ILC3) from paired PBMC and SFMC from JIA patients. (F) Representative histogram showing the protein expression of CCR6 by MFI on each ILC subpopulation (ILC1, ILC2, NCR-ILC3 and NCR+ILC3) from paired PBMC and SFMC from JIA patients. (G) Summary plot showing CCR6 positive cells as % of ILC subpopulations. (H) Summary plot showing protein expression of CCR6 by MFI on ILC subpopulations. Statistical analysis carried out by Kruskal – Wallis or by paired T test. Bars represent median values * $p<0.05$

5.2.2 Cytokines at the inflamed site may play a role in ILC phenotype switching

Evaluation of chemokines and chemokine receptors suggested that selective migration and recruitment of subpopulations of ILC may contribute to ILC recruitment into the inflamed joint in patients with JIA. Whilst selective recruitment could explain some ILC subpopulation frequencies within SFMC, ILC are also known to exhibit plasticity dependent on local cytokine milieu (Hazenberg and Spits, 2014b). In order to test the hypothesis that ILC undergo phenotype switching within the inflamed site in the joints of patients with JIA, cytokines in the SF from JIA patients were measured by multiplex cytokine analysis, and the influence of specific cytokines on ILC phenotype was analysed *in vitro* culture studies.

IL-1β, IL-2, IL-6, IL-7, IL-10, IL12, IL-13, IL-15, IL-17A, IL-17F, IL-18, IL-22, IL-23, IL-25, IL-27, IL-33, IFNα, IFNβ, IFNγ, GM-CSF, TNFα and TSLP were analysed in paired blood serum and SF samples from 16 JIA patients by multiplex cytokine analysis, in collaboration with Dr W. de Jager, University of Utrecht using the Luminex platform (de Jager et al., 2007). The dynamic range of the Luminex assay is ~5pg/ml-10,000pg/ml. For concentrations above this range, values were generated by extrapolation of the standard curve. Concentrations below the lower limit of detection were allocated a concentration of 0pg/ml, however the true value is likely to be above zero.

Significantly higher concentrations of IL-1β, IL-6 and IL-15 were demonstrated in the SF compared to serum protein levels (Figures 5.6A-C). In this set of 16 samples, and in contrast to previous data, IL-17A and associated cytokines IL-17F, IL-23, IL-22 and GM-CSF were not detected in higher concentrations in the SF compared to serum although some individual SF samples showed high levels (Figures 5.6D-H). No differences were seen between SF and serum for any other cytokines analysed (Figure 5.6I-V). There was significant variability in cytokine protein levels between samples and a larger cohort analysis would be required to be adequately powered to draw firm conclusions.


A
IL-1β
**
Concentration (pg/ml)
100
80
60
40
20
0
Blood
Synovial fluid
B
IL-6
****
Concentration (pg/ml)
60000
40000
20000
0
Blood
Synovial fluid
C
IL-15
**
Concentration (pg/ml)
150
100
50
0
Blood
Synovial fluid
D
IL-17A
Concentration (pg/ml)
600
400
200
0
Blood
Synovial fluid
E
IL-17F
Concentration (pg/ml)
8000
6000
4000
2000
0
Blood
Synovial fluid
F
GMCSF
Concentration (pg/ml)
50
40
30
20
10
0
Blood
Synovial fluid
G
IL-22
Concentration (pg/ml)
800
600
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IL-23
Concentration (pg/ml)
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Blood
Synovial fluid

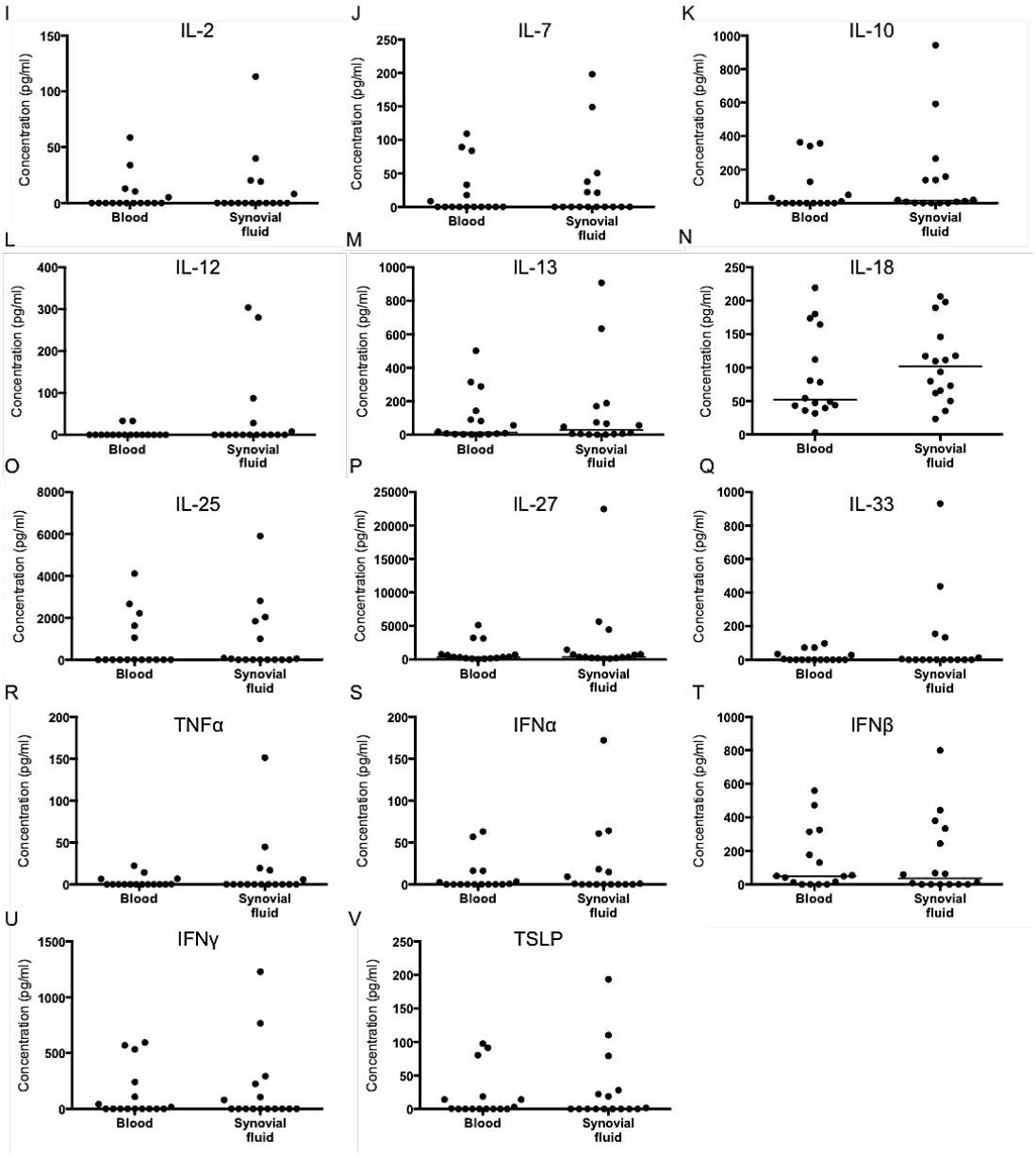

Figure 5.6 Multiplex cytokine analysis of synovial fluid and peripheral blood serum of JIA patients.

Paired JIA SF and peripheral blood serum samples (n=16), collected at time of active clinical disease when patient attend for joint injection were analysed for specific cytokine concentrations using the Luminex platform. (A-C) Summary plots showing concentrations of cytokines that were significantly different between serum and SF (A) IL-1β; (B) IL-6 and (C) IL-15. (D-H) Summary plots showing concentrations of IL-17-associated cytokines (D) IL-17A;(E) IL-17F;(F) GMCSF;(G) IL-22 and (H) IL-23. (I-V) Summary plots showing concentrations of cytokines that were not significantly different between serum and SF (I) IL-2;(J) IL-7;(K) IL-10;(L) IL12;(M) IL-13;(N) IL-18;(O) IL-25;(P) IL-27; (Q) IL-33;(R) TNFα;(S) IFNα; (T) IFNβ; (U) IFNγ and (V) TSLP. Statistical analysis carried out by paired T test. Bars represent median values. **p<0.01 ****p<0.0001.

	Serum (pg/ml) Median (IQR)	SF (pg/ml) Median (IQR)
IL-1β	0.0(0.0-0.82)	1.48(0.0-16.31)
IL-2	0.0(0.0-9.10)	0.0(0.0-16.38)
IL-6	0.0(0.0-28.58)	3484(1477-30433)
IL-7	0.0(0.0-29.14)	0.0(0.0-33.69)
IL-10	0.0(0.0-107.7)	15.36(0.77-153.1)
IL-12	0.0(0.0-0.0)	0.0(0.0-23.13)
IL-13	13.96(3.35-129.6)	29.65(4.68-146.0)
IL-15	1.27(0.0-23.97)	18.6(12.38-43.93)
IL-17A	0.0(0.0-0.0)	3.83(0.0-26.77)
IL-17F	0.0(0.0-68.81)	0.0(0.0-28.81)
IL-18	51.68(40.34-151.4)	101.7(62.91-138.8)
IL-22	0.0(0.0-0.0)	0.0(0.0-3.08)
IL-23	82.89(0.0-783)	0.0(0.0-1847)
IL-25	0.0(0.0-1479)	20.47(0.0-1625)
IL-27	348.4(161.5-751)	358.7(213.3-1280)
IL-33	0.0(0.0-32.25)	0.0(0.0-102.7)
TNFα	0.0(0.0-4.96)	0.03(0.0-14.09)
IFNα	0.0(0.0-13.0)	0.42(0.0-17.26)
IFNβ	49.57(3.12-279.9)	37.74(0.0-311.4)
IFNγ	0.0(0.0-207.8)	0.0(0.0-194.6)
TSLP	0.4(0.0-17.45)	0.1(0.0-26.55)
GM-CSF	0.0(0.0-1.13)	0.0(0.0-3.55)

Table 5.2 Cytokine concentrations in serum and SF from JIA patients

Based on the results of the multiplex cytokine analysis, the effect of cytokines on ILC phenotype was investigated by culturing ILC *in vitro* in the presence of recombinant human (rh) cytokines that promote specific differentiation (n=5). Briefly, total ILC (CD45+lineage-CD127+CD161+) were sorted by flow cytometry from aHC (n=5) (as described in Chapter 2) and cultured for 7 days with (a) IL-2 alone (medium), or IL-2 medium with (b) IL-1β and IL-23 (known to skew towards ILC3 populations (Bernink et al., 2015)); or (c) IL-1β, IL-23 and IL-6; or (d) IL-6 (in order to test the hypothesis that IL-6 may drive ILC plasticity). At the end of 7-day culture *in vitro,* cells were firstly assessed for viability and proliferative activity. ILC were >80% viable (as determined by staining with a live dead dye) under all culture conditions (Figure 5.7A and 5.7B). ILC at the start of culture had very low proportion of cells in active proliferation as measured by Ki67 expression. However, cells proliferated significantly during culture in all conditions ($p>0.0001$) (Figures 5.7C and 5.7D).

Next, I investigated the impact of the skewing conditions on the ILC subpopulations, for this purpose, expression of ILC markers CRTH2, cKit and NKp44 was analysed before and after culture (Figure 5.8A, B). After culture, the ILC2-specific surface protein, CRTH2, could not be detected. This made it impossible to accurately determine ILC subpopulation on day 7, since it was not known whether this resulted from downregulation of CRTH2, or from true phenotype switching (Figure 5.8A).

cKit+NKp44- ILC, which in this context may represent NCR-ILC3 subpopulation, were significantly increased after culture with IL-6 alone compared to ILC cultured under other cytokine conditions (Figure 5.8C). None of the culture conditions significantly affected NKp44 expression; however a small increase of cKit+NKp44+ cells were seen after culture in the presence of IL-1β and IL-23, or IL-1β, IL-23 and IL-6 conditions, compared to cells cultured with IL-2 alone, which could represent skewing of ILC cells *in vitro* towards the NCR+ILC3 population (Figure 5.7D). Treatment with PMA, Ionomycin and Brefeldin A on day 7 resulted in significant cell death and it was therefore not possible to accurately determine cytokine production by ILC after these cultures (data not shown).

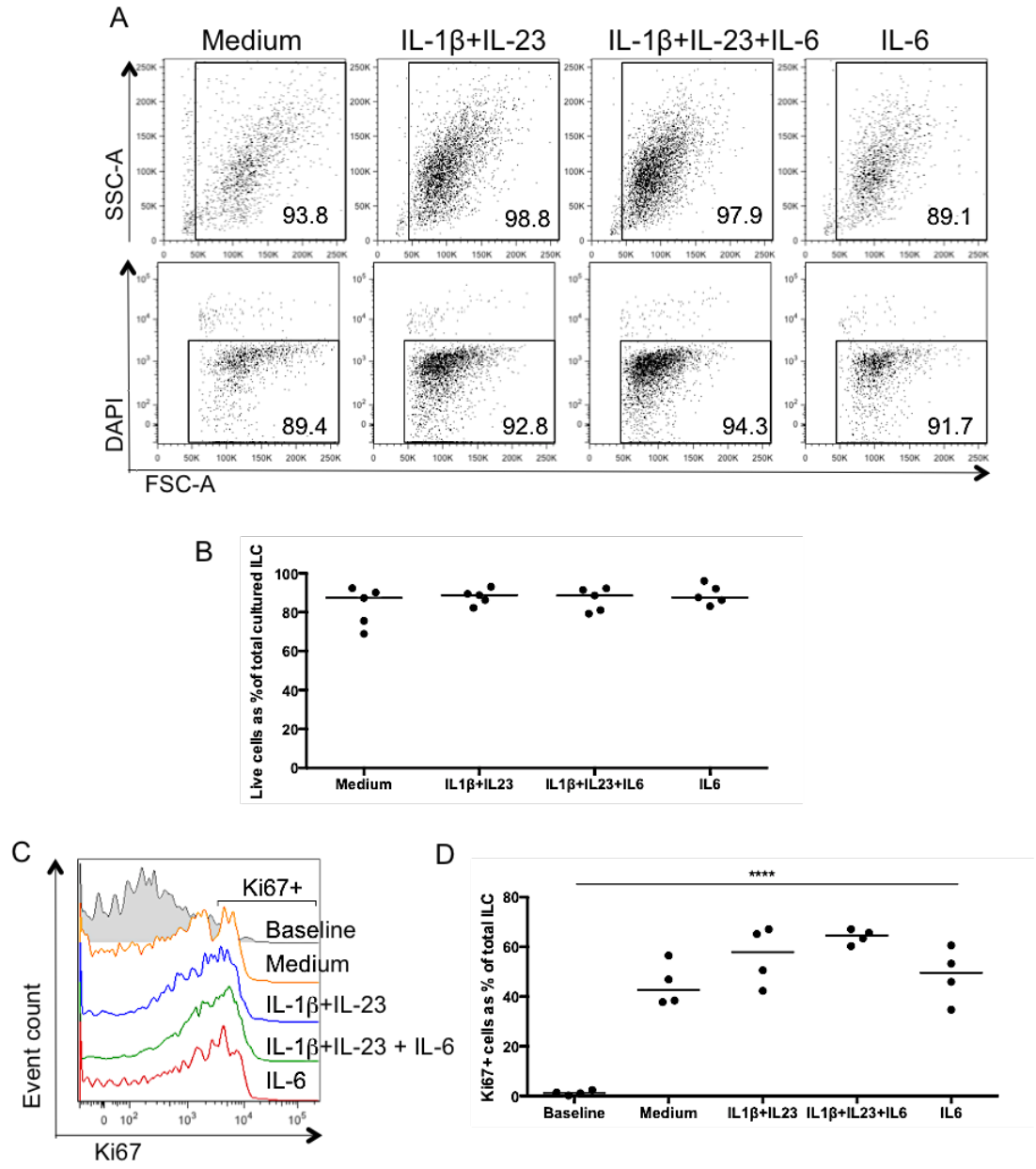

Figure 5.7 ILC survive 7-day culture and proliferate.

FACS-sorted total ILC (CD45+lineage-CD127+CD161+) from aHC PBMC (n=5) were cultured for 7 days in the presence of IL-2 alone, or IL-2 with skewing cytokine combinations (IL-23 and IL-1β; IL-23, IL-1 β and IL-6; or IL-6) as shown. (A) Representative plots showing scatter profiles (top row) and viability staining (bottom row) of ILC after culture under various skewing environments. (B) Summary plot showing live cells as % of total cultured cells on day 7 of culture. (C) Representative histogram showing intracellular Ki67 expression by ILC before (baseline) and after culture (conditions as shown). (D) Summary plot showing Ki67+ cells as a % of total live ILC *ex vivo* and after 7 days culture with skewing cytokines. Statistical analysis carried out by Kruskal -Wallis. Bars represent median values. **** p<0.0001.

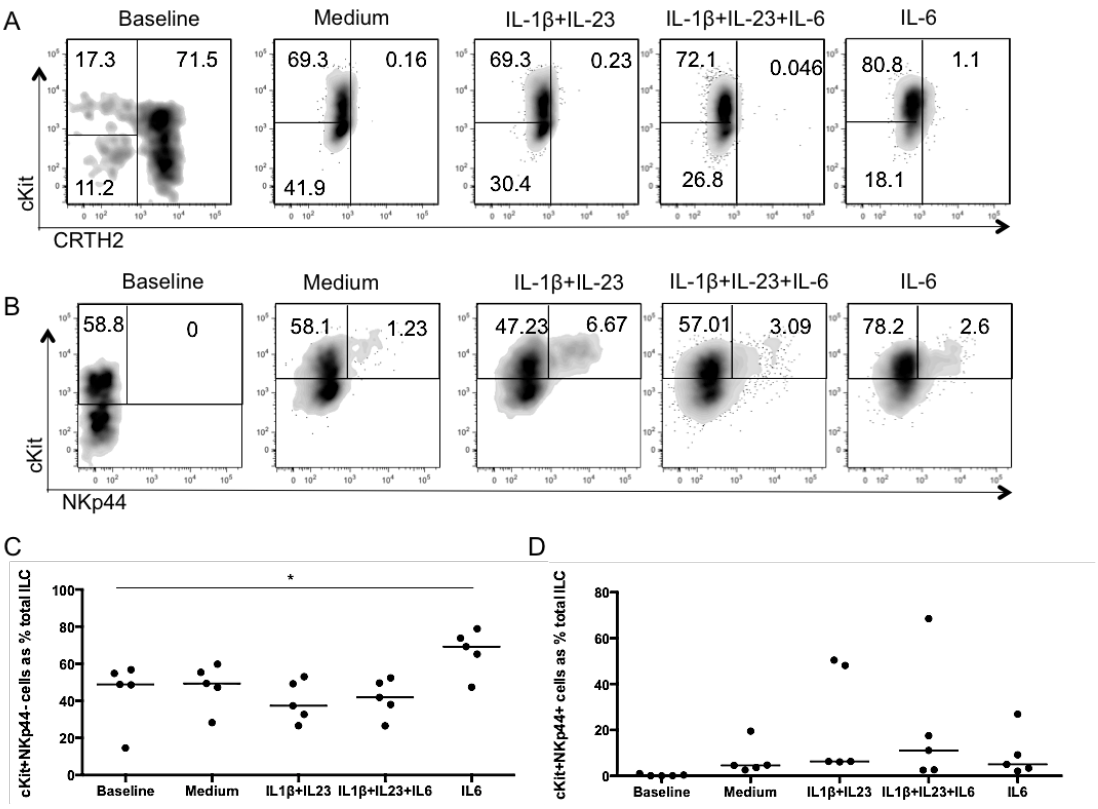

Figure 5.8 CRTH2 is down-regulated on cultured ILC.

FACS-sorted total ILC (CD45+lineage-CD127+CD161+) from aHC PBMC (n=5) were cultured for 7 days in the presence of IL-2 alone, or IL-2 with skewing cytokine combinations (IL-23 and IL-1β; IL-23, IL-1 β and IL-6; or IL-6) as shown. (A) Representative plots showing CRTH2 expression on *ex vivo* ILC and ILC after 7 days culture. (B) Representative plots showing expression of cKit and NKp44 by *ex vivo* ILC and ILC after 7 days culture with skewing conditions. (C) Summary plot showing cKit+NKp44- ILC before (baseline) and after culture (conditions as shown), and (D) Summary plot showing cKit+NKp44+ ILC before and after culture. Statistical analysis carried out by Kruskal -Wallis. Bars represent median values. * $p<0.05$.

While the *in vitro* culture experiments revealed some change in phenotype of ILC, it was important to attempt to mimic the synovial inflamed environment more closely in these experiments. Therefore to investigate the physiological effect of synovial fluid on ILC phenotype, total ILC (CD45+lineage-CD127+CD161+) were again FACS sorted from aHC PBMC and cultured for 7 days with IL-2 alone (medium) or IL-2 with 50% synovial fluid (n=4). Given the observed heterogeneity of concentrations of different cytokines and chemokines within the 16 SF samples (Table 5.2) a strategy of a pooled sample of mixed SF was applied.

A pool of synovial fluid was made by combining synovial fluid samples from 10 JIA patients. Cytokine protein levels in the SF pool were measured by multiplex cytokine analysis as described above. Significant amounts of IL-6 (7.23µg), IL-25 (2.09ng/ml), IL-27 (0.013µg/ml) and IFNβ (1.2ng/ml) were detected (Figure 5.9A). All cytokine concentrations from the SF pool are shown in Table 5.3. In relation to the *in vitro* system used in initial cultures above, the synovial pool contained a significantly higher concentration of IL-6, but lower concentrations of IL-1β and IL-23. Of note, although IL-2 concentrations in the SF pool were relatively low (5.17pg/ml), all cultures contained additional IL-2, supplemented at a final concentration of 0.5ng/ml as above. The pool of SF was used at a concentration of 50%. The actual culture concentrations of each cytokine were as shown in left column of Table 5.3.

ILC after culture in IL-2/50% SF medium (Figure 5.8) remained >60% viable after 7 days (Figure 5.9B and 5.9C). Interestingly, higher proliferation was seen from samples cultured with 50% SF compared to IL-2 alone (Figure 5.9D). As seen previously, a down regulation of ILC2 marker CRTH2 was observed (Figure 5.9E). In contrast, IL-2/50% SF did not affect cKit or NKp44 ILC expression compared to cells cultured with IL-2 alone (Figure 5.9F, 5.9G and 5.9H). Low cell numbers after culture meant it was not possible to accurately assess the cytokine production by ILC on day 7 (data not shown).

Together, these results identify the presence of cytokines at the inflamed site that are known to play a role in ILC plasticity. The culture assays performed do not conclusively demonstrate ILC plasticity *in vitro,* but may suggest an effect of IL-6 on the ILC3 population at the inflamed site.

	Concentration in SF pool (pg/ml)	**Final concentration used in cultures (pg/ml)**
IL-1β	141	70.5
IL-2	5.17	2.585
IL-6	7232674	3616337
IL-7	0	0
IL-10	40.79	20.395
IL-12	43.07	21.535
IL-15	33.32	16.66
IL-17A	19.8	9.9
IL-22	19.5	9.75
IL-23	105.64	52.82
IL-25	2087.31	1043.655
IL-27	12762.34	6381.17
TNFα	1.26	0.63
IFNα	13.88	6.94
IFNβ	1202.84	601.42
IFNγ	0	0
GM-CSF	19.93	9.965

Table 5.3 Concentration of cytokines within the synovial fluid pool

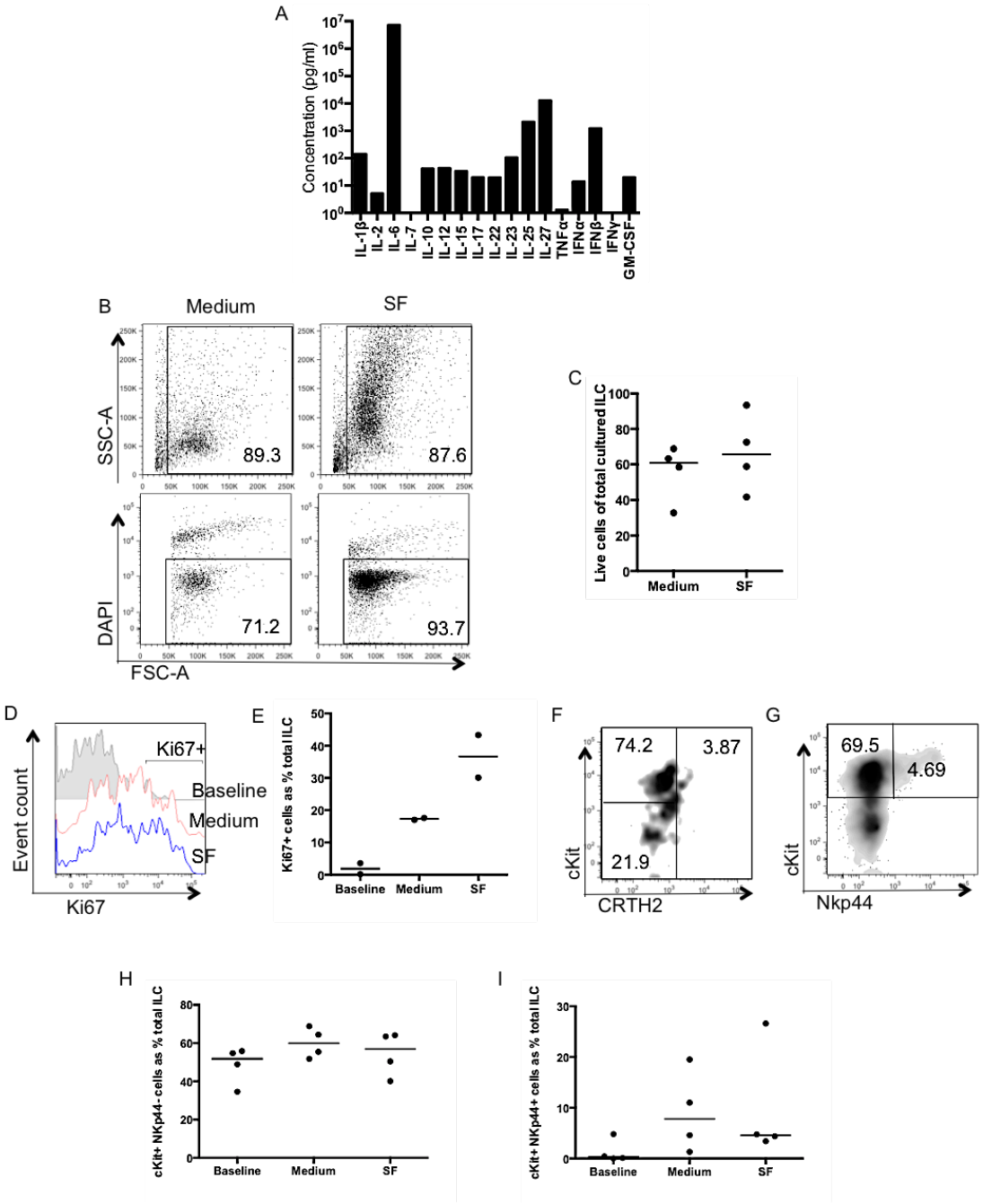

Figure 5.9 Synovial fluid does not cause skewing of ILC after 7 days.

FACS- sorted total ILC (CD45+lineage-CD127+CD161+) from aHC PBMC (n=4) were cultured for 7 days in the presence of IL-2 alone or IL-2 plus 50% SF from a pool made by combining SF from 10 JIA patients. (A) Multiplex cytokine analysis of cytokines in the SF pool used for culture by Luminex. (B) Representative plots showing scatter profiles (top row) and viability staining (bottom row) of ILC after culture under with IL-2 medium alone (left panels) or 50% SF (right panels). (C) Summary plot showing live cells as % of total cultured cells on day 7 of culture. (D) Representative histogram showing Ki67 expression on ILC before and after culture. (E) Ki67+ cells as a % of total live ILC *ex vivo* and after 7 days culture. Representative plots showing (F) CRTH2 expression and (G) expression of cKit and NKp44 on ex vivo ILC and ILC after 7 days culture with 50% SF. Summary plots showing (H) cKit+NKp44- ILC and (I) cKit+NKp44+ ILC before and after culture.

5.2.3 Myeloid DC are enriched at the inflamed site and correlate with NCR+ILC3

During routine analyses of SFMC cellular composition, an enrichment of myeloid dendritic cells (mDC) was noted, relative to DC frequency in PBMC (Chapter 3, Figure 3.2). These observations raised a new hypothesis that mDC at the inflamed site may produce cytokines or other inflammatory mediators, which could contribute to ILC phenotype or mediate ILC switching from one subpopulation to another in the joint. In order to investigate this hypothesis, the phenotype of synovial mDC was examined and compared to patient and adult a child control healthy mDC from the PBMC compartment. Cells were gated on CD14 and CD11c and analysis performed by flow cytometry (after exclusion of dead cells) (Figure 5.10A).

A significant enrichment of mDC (defined as CD14-CD11c+ cells) was confirmed in the SFMC from JIA patients (n=34) compared to patient PBMC (n=13) or adult (n=12) or child (n=3) healthy controls ($p<0.0001$) (Figure 5.10A and 5.10B). When synovial mDC were analysed against the relative frequency of SFMC ILC3 cells in the same patient, significant correlations were observed. mDC showed a strong negative correlation with NCR-ILC3 at the inflamed site ($r=-0.73$, $p= <0.0001$) (Figure 5.10C). Conversely, mDC were strongly positively associated with NCR+ILC3 ($r=0.67$, $p=0.0005$) (Figure 5.10D). Interestingly, the correlation between mDC and ILC3 was not maintained in the PBMC from JIA patients (Figure 5.10E), supporting a possible shared mechanism or interactions between mDC and ILC3 populations at the inflamed site.

Additionally, the number of synovial mDC within the SFMC showed weak negative correlations with measures of disease severity at the time of synovial fluid sampling, including ESR ($r=-0.47$, $p=0.067$) and Physician's VAS ($r=-0.33$, $p=0.13$) (Figure 5.11B and 5.11C). No associations were seen between synovial mDC and active joint count, or JADAS3 scores at the time of sample (Figures 5.11A and 5.11D).

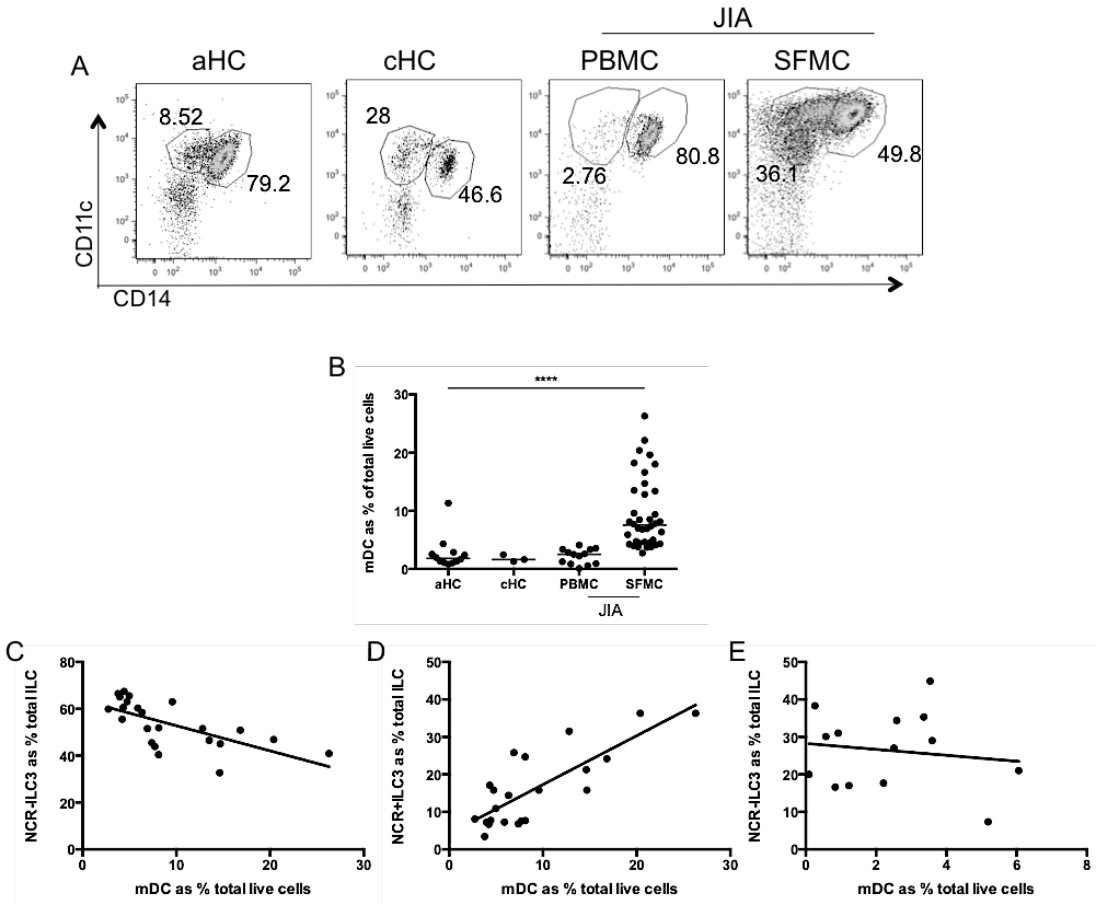

Figure 5.10 mDC are enriched at the inflamed site and correlate with ILC3 populations.

(A) Representative plots showing the gating strategy for the enumeration of mDC (CD14-CD11c+) in the PBMC from aHC and cHC and PBMC and SFMC from JIA patients. (B) Summary plot showing mDC in PBMC of aHC (n=12) and cHC (n=3) and PBMC (n=13) and SFMC (n=34) of JIA patients. (C-D) Correlations between mDC in the SFMC of JIA patients analysed against (C) NCR-ILC3 (n=23, r=-0.73, p<0.0001) and (D) NCR+ILC3 (n=23, r=0.67, p=0.0005). (E) Correlation between mDC and NCR-ILC3 from within the PBMC of JIA patients (n= 14, r=-0.015, p=0.96). Statistical analysis carried out by Kruskal -Wallis. Bars represent median values. * p<0.05. Correlations by Spearman correlation.

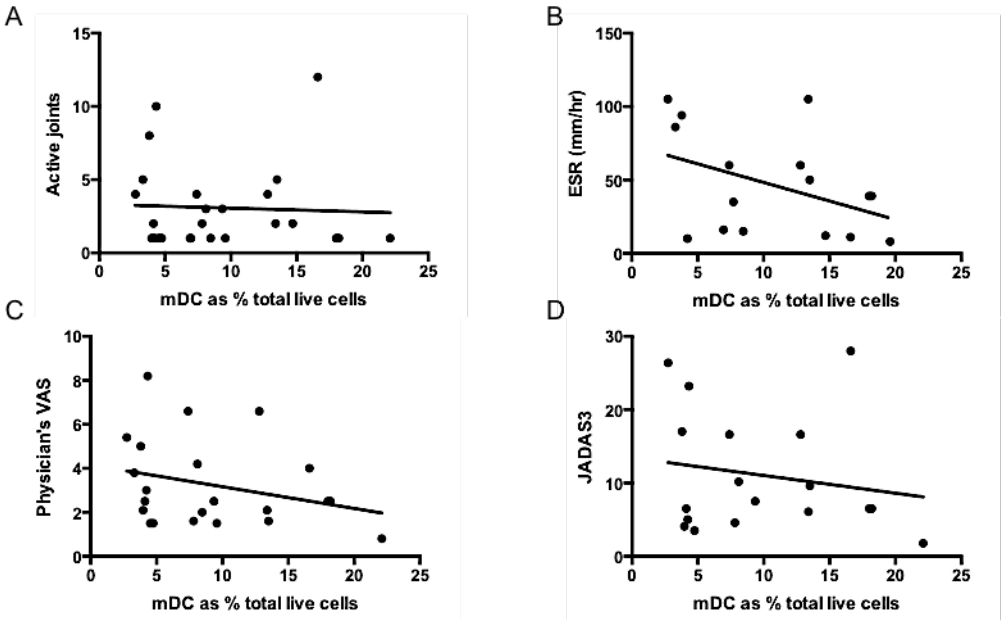

Figure 5.11 mDC are associated with less severe disease as estimated by ESR and Physician's VAS.

(A-D) Correlations in the SFMC of JIA patients showing mDC subpopulations as a % of total live cells analysed against clinical measures of disease severity: (A) Analysed against number of active joints at the time of sample (n=25, r=-0.15, p=0.47); (B) Analysed against ESR at time of sample (n=16, r=-0.47, p=0.067); (C) Analysed against physician's VAS (n=22, r=-0.33, p=0.13) and (D) Analysed against JADAS3 (n=18, r=-0.17, p=0.50). Correlations by Spearman correlation.

5.2.4 Synovial mDC express low cytokine mRNA transcript levels

In order to investigate the putative interaction of mDC with ILC populations at the inflamed site, the synovial mDC phenotype and functional capacity was investigated by qPCR. Magnetic bead sorting and FACS sorting protocols were tested in order to ascertain the optimal sorting strategy for mDC (Chapter 2). Magnetic bead sorting (StemCell) using negative selection resulted in relatively impure mDC isolations (40-80% purity) and was therefore deemed unsuitable for further use (Figure 5.12A and 5.12B). In contrast, highly pure mDC samples (>95% purity) could be isolated by FACS sorting based on expression CD11c and CD14 (Figure 5.12C and 5.12C). mDC were FACS sorted from 3 aHC PBMC and 3 JIA SFMC samples and RNA was extracted for subsequent qPCR experiments.

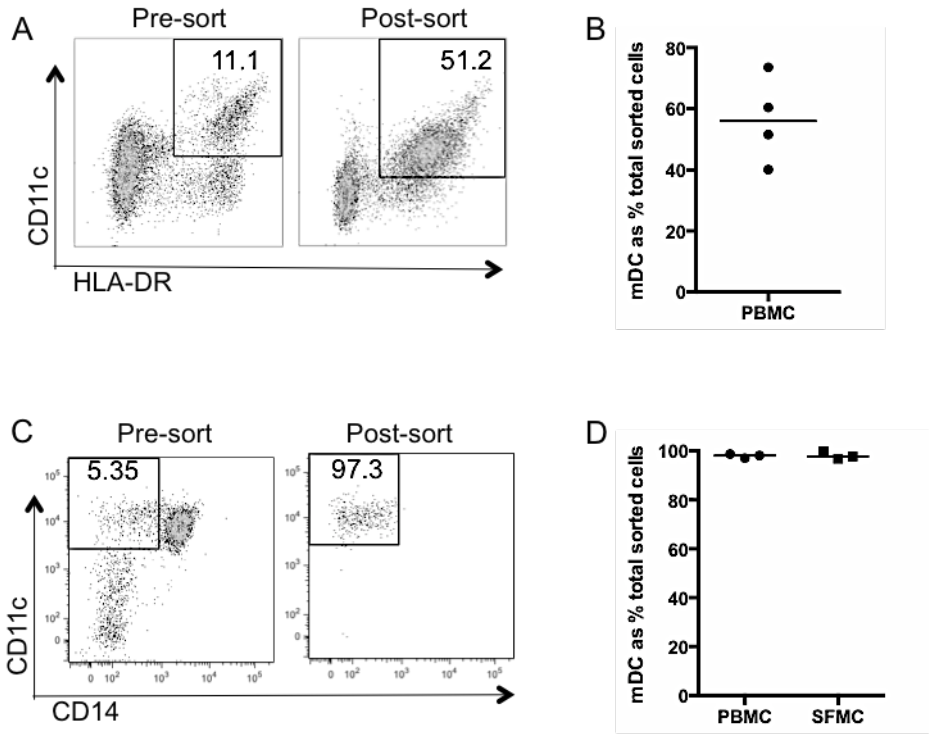

Figure 5.12 Flow cytometric sorting is more efficient than magnetic bead isolation for the isolation of mDC from blood and SFMC.

Magnetic bead sorting and flow cytometric sorting techniques were tested and compared in order to ascertain the optimal sorting strategy for mDC from aHC PBMC. (A) Representative plots showing mDC proportions before and after sorting by EasySep magnetic bead separation. (B) Summary plot showing mDC purity after magnetic bead separation from PBMC (n=4). (C) Representative plots showing mDC gating strategy (CD14-CD11c+) and purity after flow cytometric sorting. (D) Summary plot showing mDC purity after flow cytometric sorting from PBMC (n= 3).

In order to test the hypothesis that mDC at the inflamed site may contribute to the cytokine signature seen at the inflamed site, and may influence the ILC phenotype in the joint by the production of skewing cytokines, the cytokine profiles of mDC (CD11c+CD14-) were assessed by qPCR for the gene expression of *IL1β, IL23* and *IL6* mRNA transcripts. As a positive control healthy sorted mDC from healthy adult donor was stimulated with LPS (100ng/ml for 15 hours) prior to RNA extraction. Data were analysed using a standard curve generated from the LPS stimulated mDC cDNA and samples were normalized using housekeeping gene *ACTB*.

Surprisingly, relative expression of *IL1β, IL23* and *IL6* genes were all lower in mDC isolated from JIA SFMC compared to aHC PBMC (Figure 5.13). However, the difference did not reach significance and sample numbers were small meaning that these data should be interpreted with caution. When compared to gene expression in LPS-stimulated mDC from a healthy control, gene expression from both PBMC and SFMC was low for all cytokines measured.

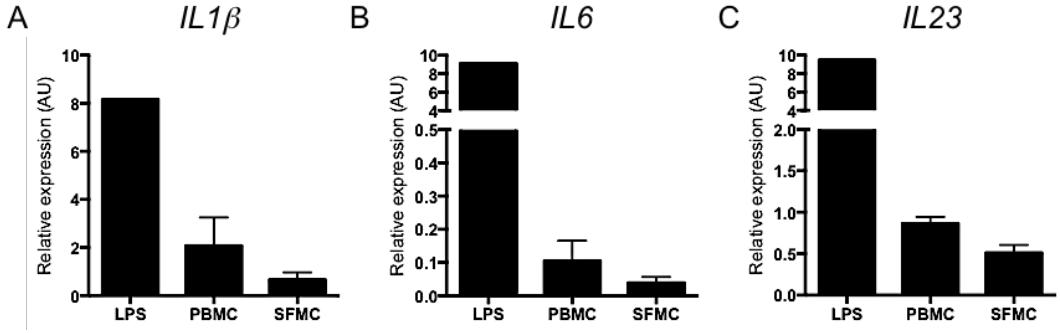

Figure 5.13 mDC in SFMC express low levels mRNA transcripts for cytokine genes.

qPCR was performed to amplify specific transcripts from cDNA generated from mDC sorted by flow cytometry from JIA SFMC (n=3) and aHC PBMC (n=3), and from aHC sorted mDC subsequently stimulated with LPS (n=1) (100ng/ml for 15 hours). Relative expression of (A) *IL1β* (B) *IL6* and (C) *IL23.* Bars represent the mean value of the 3 mDC samples, error bars represent SEM.

5.2.5 Synovial mDC express higher CD103 and produce retinoic acid

More detailed phenotyping of the DC populations at the inflamed site was then conducted. For the purposes of this analysis, four populations were considered namely total mDC (CD11c+CD14-), BDCA1+(CD1c) mDC, BDCA3+ (CD141) mDC and pDC (CD123+BDCA2+). In these experiments, a 'lineage dump' channel was used, which included antibodies to CD3, CD34, CD19 and CD56 to exclude T cells, stem cells, B cells and NK cells.

BDCA1+, BDCA3+ and pDC subpopulations were enumerated in healthy control PBMC of adults and children, PBMC and SFMC from JIA patients (Figure 5.14A and B). BDCA1+ and BDCA3+ cells as a % of total live cells were enriched in the SFMC of patients compared to paired JIA, or healthy, PBMC ($p<0.0001$) (Figures 5.14C and 5.14D). pDC were also enriched in the SFMC compared to PBMC of patients ($p=0.041$) (Figure 5.14E).

Since mDC in the SFMC appeared to express relatively low levels of cytokines IL-1β, IL-6 and IL-23 at mRNA level it is possible that they were in a 'tolerogenic' state and may play a role in promoting regulation at the inflamed site. To test this hypothesis, mDC from both PBMC and SFMC were analysed for expression of CD103 (also known as αE integrin), a surface protein associated with a population of tolerogenic DC which are able to produce retinoic acid, RA, which has recently been shown to promote ILC switching towards NCR+ILC3 (Coombes et al., 2007, Bernink et al., 2015) (Figure 5.15A). Synovial mDC contained a significantly higher proportion of CD103 positive cells compared to mDC from patient or control PBMC in total mDC (CD11c+CD14-), BDCA1+ and BDCA3+ DC populations ($p<0.0001$, $p<0.0001$ and $p=0.0004$ respectively) (Figures 5.15B-D).

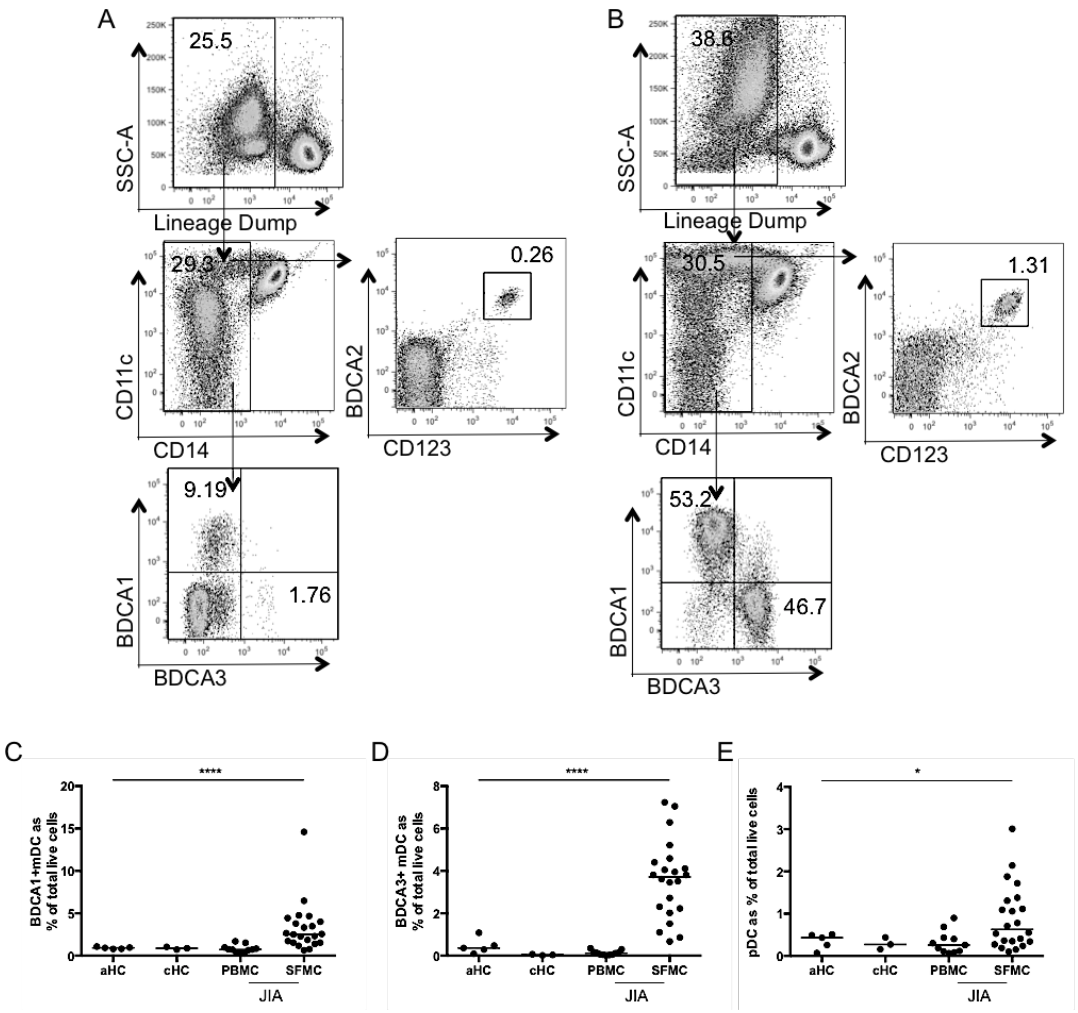

Figure 5.14 BDCA1+ and BDCA3+ mDC and pDC are enriched at the inflamed site in JIA.

BDCA1+, BDCA3+ and pDC were enumerated in the PBMC from aHC (n=5) and cHC (n=3) and PBMC (n=11) and SFMC (n=21) from JIA patients by flow cytometry. Representative plots showing the gating strategy for the identification of DC subsets in (A) PBMC and (B) SFMC of JIA patients. Summary plots showing DC subsets at a % of total live cells for (C) BDCA1+ mDC; (D) BDCA3+mDC and (E) pDC. Statistical analysis carried out by Kruskal -Wallis. Bars represent median values. *$p<0.05$ ****$p<0.0001$

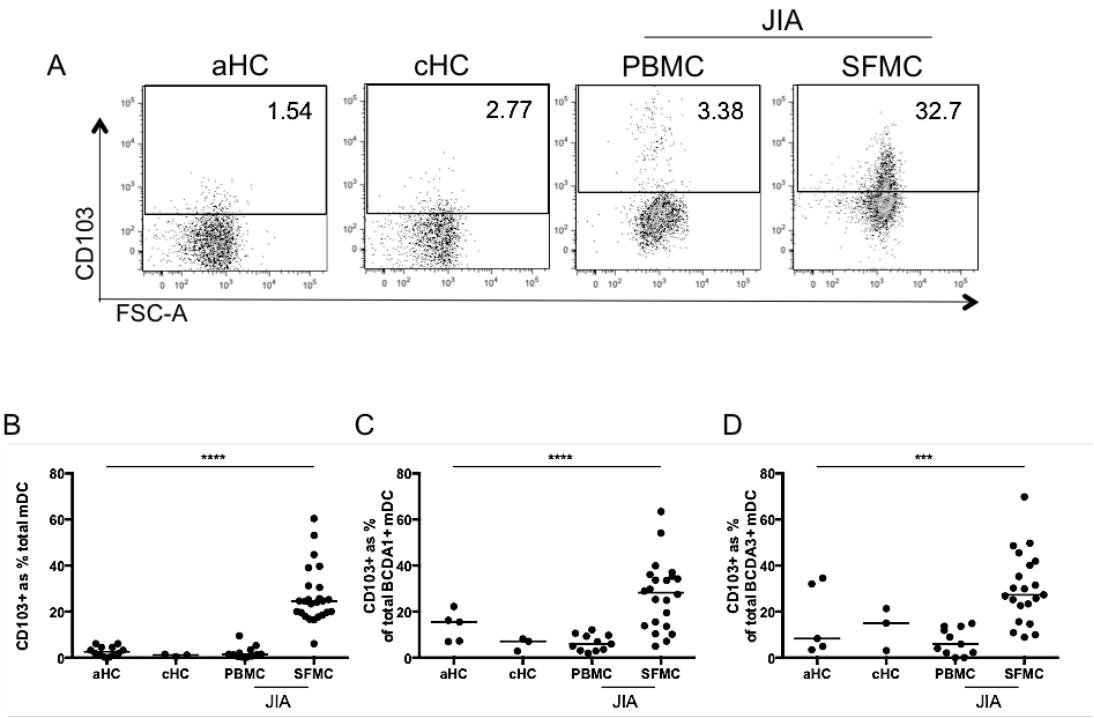

Figure 5.15 CD103+mDC are enriched at the inflamed site in JIA.

CD103+ mDC were enumerated in the PBMC from aHC (n=12) and cHC (n=3) and the PBMC (n=12) and SFMC (n=25) from JIA patients by flow cytometry. (A) Representative plots showing the gating strategy for the identification of CD103+ cells in the PBMC and SFMC of JIA patients. Previously gated on mDC (CD14-CD11c+) as shown in Figure 5.10. Summary plots showing CD103+ cells at a % of mDC subsets for (B) total mDC (CD14-CD11c+) (C) BDCA1+ mDC; (D) BDCA3+mDC. Statistical analysis carried out by Kruskal -Wallis. Bars represent median values. ***p<0.001****p<0.0001.

The ability of mDC from the joint to produce RA was then tested *in vitro,* using the Aldefluor assay (Hurst and Else, 2013) as described in Chapter 2 . This is a flow cytometry-based assay that measures the amount of active aldehyde dehydrogenase (ALDH) enzymes in each cell, as a surrogate of RA production. A control reaction was run for each sample including the DAEB inhibitor, which prevents the active aldefluor substrate from binding to the ALDH enzyme. When aldefluor binds the ALDH enzyme fluorescence is detected in the FITC channel. The negative gate was set according to the DAEB control and was specific for each sample. Positive events were detected within this gate (Figure 5.16A). Interestingly among control PBMC samples there was an apparent age difference in that adult mDC contained on average more cells that have active ALDH enzyme than mDC from healthy children.

Significantly higher proportions of ALDH+ mDC were identified in SFMC compared to PBMC samples of either healthy children or JIA patients (Figure 5.16B). Given the strong enrichment for CD103+ mDC in the SFMC population, the relative difference in cells with ALDH activity was assessed between CD103+ and CD103- mDC from the SFMC of JIA patients (Figure 5.17A). A small but significantly higher ALDH activity, expressed as % of cells positive for RALDH in the Aldefluor assay, was detected in CD103+ mDC compared to the CD103- mDC within SFMC samples ($p=0.016$) (Figure 5.17B).

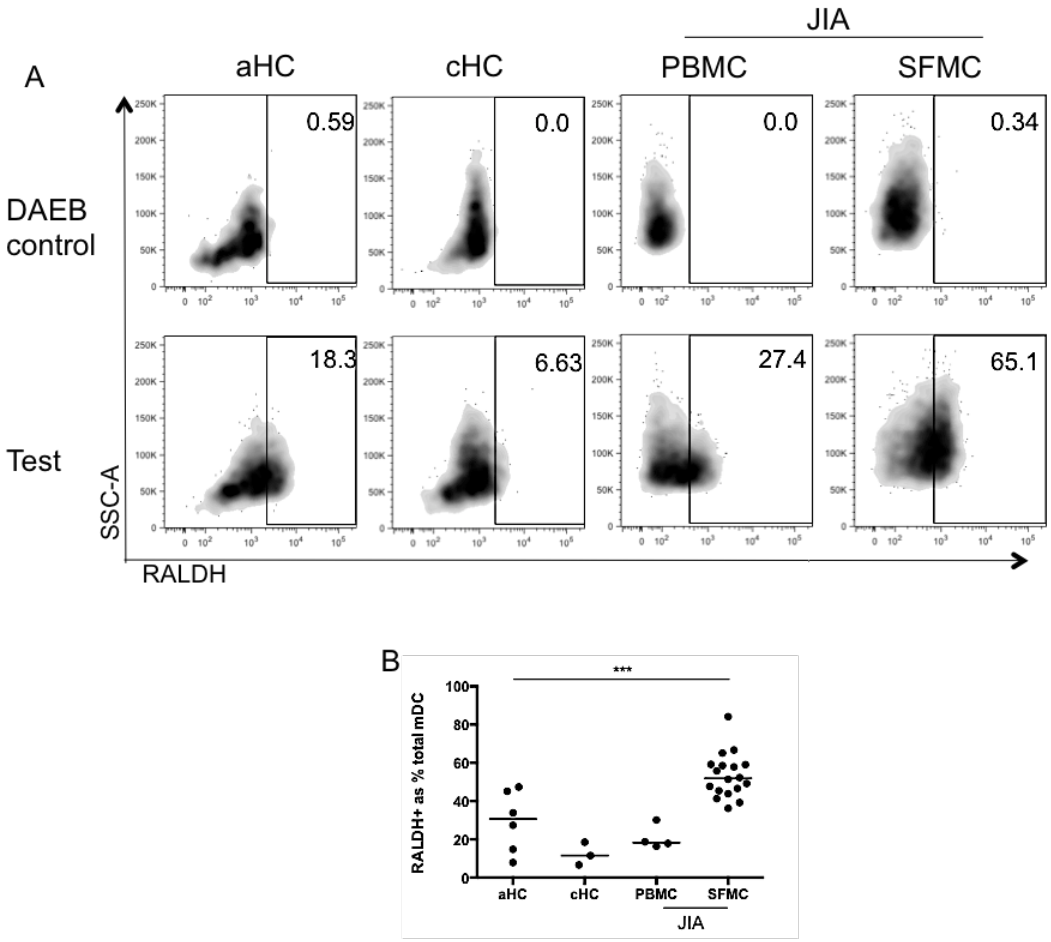

Figure 5.16 RALDH+ mDC are enriched in the joints of JIA patients.

RALDH expression was analysed within mDC (CD14-CD11c+) by flow cytometry in the PBMC from aHC (n=6) and cHC (n=3) and the PBMC (n=4) and SFMC (n=18) from JIA patients. (A) Representative plots showing the gating strategy. The positive gate was set according to DEAB negative control (top row). Positive events were detected within this gate (bottom row). Previously gated on CD14-CD11c+mDC. (B) Summary plots showing RALDH+ cells as a % of total mDC. Statistical analysis carried out by Kruskal - Wallis. Bars represent median values. ***p<0.001

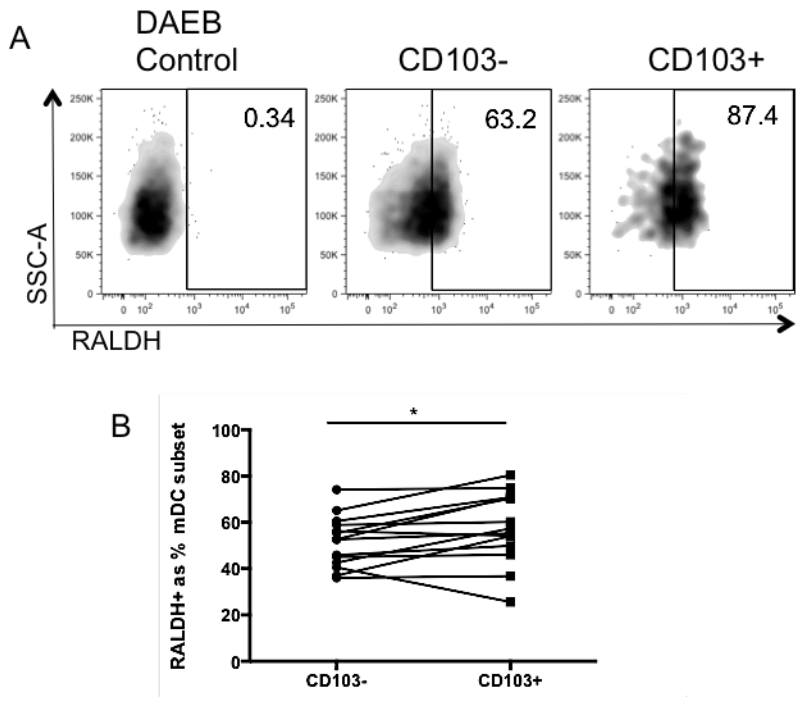

Figure 5.17 RALDH expression is higher in CD103+mDC at the inflamed site.

RALDH expression was analysed by flow cytometry on CD103+ and CD103- mDC populations from the SFMC of JIA patients (n=14). (A) Representative plots showing the gating strategy for identification of RALDH+ cells. Previously gated on CD14-CD11c+mDC. (B) Summary plot showing the relative RALDH expression in CD103- and CD103+mDC. Statistical analysis carried out by paired T test. Bars represent median values. * $p<0.05$

To investigate RALDH mRNA expression in PBMC and SFMC mDC, *ALDH1A1* and *ALDH1A2*, qPCR was performed on FACS-sorted mDC from aHC (n=3) and JIA SFMC (n=3). While a trend towards higher *ALDH1A1* gene expression was seen in aHC mDC compared to SFMC mDC, the opposite was true for *ALDH1A2,* which was more highly expressed in SFMC mDC (Figures 5.18A and 5.18B).

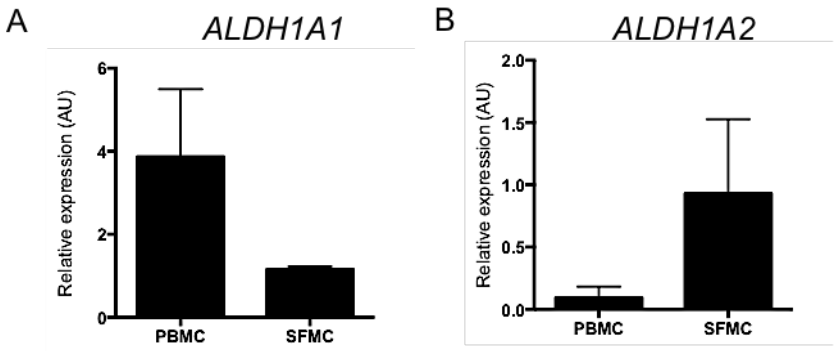

Figure 5.18 mDC in SFMC show differential expression of ALDH1A1 and ALDH1A2 gene transcripts compared to mDC from aHC.

qPCR was performed to amplify specific transcripts from cDNA generated from mDC (CD14-CD11c+) FACS sorted from JIA SFMC (n=3) and aHC PBMC (n=3). Relative expression of (A) *ALDH1A1* and (B) *ALDH1A2.* Bars represent the mean value of the 3 mDC samples, error bars represent SEM.

5.2.6 Synovial ILC have increased expression of retinoic acid receptor gene *RARA*

The ability of synovial fluid ILC to respond to RA was next investigated to support the hypothesis that RA production from mDC at the inflamed site may be involved in ILC phenotype switching towards the NCR+ILC3 population seen in the SFMC.

The chemokine receptor CCR9 gene is a direct target of RA signalling (Ohoka et al., 2011). In order to investigate the response of ILC to RA, the expression of a RA-responsive gene product, CCR9 on ILC from PBMC from aHC and PBMC and SFMC from JIA patients, was examined *ex vivo* by flow cytometry. Surprisingly, CCR9 expression on ILC was relatively low on all samples analysed and was not significantly higher on synovial ILC compared to ILC from healthy or JIA PBMC (Figure 5.19).

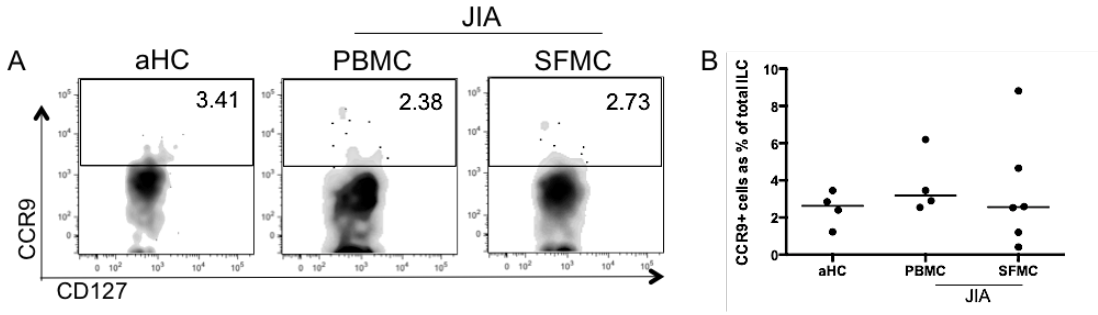

Figure 5.19 Synovial ILC do not express higher CCR9 compared to ILC from PBMC

Expression of chemokine receptor CCR9 measured on total ILC (CD45+lineage-CD127+CD161+) from aHC PBMC (n=4), and PBMC (n=4) and SFMC (n=6) from JIA patients. (A) Representative plots showing the gating strategy for the identification of CCR9 positive cells as a % of total ILC (B) Summary plot showing CCR9 positive cells as % total ILC.

Finally, gene expression of RA receptors *RARA, RARB* and *RARG* were analysed in FACS sorted ILC from aHC and JIA SFMC (n=3). A qPCR standard curve was generated from the sorted ILC with the highest gene expression during initial testing and all samples were normalized using housekeeping gene *ACTB* expression. Of the 3 RA receptor genes, only *RARA* was satisfactorily amplified in all the isolated ILC samples and was therefore the only gene that could be analysed (*RARB* and *RARG* data not shown). There was a trend towards higher *RARA* mRNA expression in ILC isolated from SFMC compared to aHC although this did not reach significance (Figure 5.20).

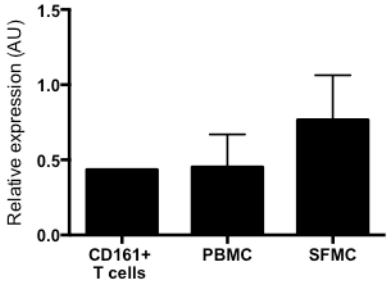

Figure 5.20 Synovial ILC show increased expression of retinoic acid receptor gene RARA.

qPCR was performed to amplify specific transcripts from cDNA generated from ILC (CD45+lineage-CD127+CD161+) FACS sorted from JIA SFMC (n=3) and aHC PBMC (n=3) and from CD161+ T cells sorted from aHC PBMC (n=1). Relative expression of *RARA.* Bars represent the mean value of the 3 ILC samples, error bars represent SEM.

5.3 Discussion

The data in Chapter 4 clearly demonstrated an altered ILC phenotype in the joint compared to the blood from JIA patients or healthy controls. This Chapter aimed to elucidate the mechanisms by which the SFMC ILC phenotype is achieved at the inflamed site, by investigating mechanisms of ILC recruitment and factors that may drive potential ILC phenotype switching within the inflamed site.

Multiplex analysis of chemokines in the blood and SF from JIA patients showed significantly higher concentrations of CCL2, CCL20, CXCL8, CXCL9 and CXCL10 at the inflamed site in this patient cohort. These findings are supported by previously published data, which have reported an abundance of these migratory ligands in JIA (Pharoah et al., 2006, de Jager et al., 2007).

Differential expression of chemokine receptors was detected on synovial ILC compared to paired blood with a significant increase of CXCR3 and CCR5 positive ILC at the inflamed site. Furthermore, ILC subpopulation-specific expression patterns were observed. Expression of both of these receptors was largely restricted to the ILC1 population. Both CXCR3 and CCR5 are often associated with Th1 cells and so the expression of these receptors on the ILC1 subpopulation was unsurprising (Odum et al., 1999). Additionally, the data obtained mirrors the enrichment of CCR5/CXCR3+ Th1 cells previous described in JIA (Wedderburn et al., 2000a). These findings are also supported by recently published data, which has described increased CXCR3 and CCR5 expression on the ILC1 subpopulations in systemic sclerosis (Roan et al., 2016a). Together these findings support a role for selective migration of ILC1 subpopulations to the inflamed site in JIA, possibly migrating to a chemokine gradient.

As previous studies have described an enrichment of CCR4+ T cells at the inflamed site in JIA (de Kleer et al., 2004, Nistala et al., 2008a), it was surprising to find reduced expression of this receptor on synovial ILC compared to paired

blood (Issekutz et al., 2011). However, this study and others have demonstrated that the ILC2 population is the dominant CCR4+ population within ILC, and therefore given the ablation of ILC2 in the inflamed joint (described in Chapter 4), the reduction of CCR4+ ILC in the joint is perhaps less surprising (Roan et al., 2016b). Furthermore, the reduced expression of CCR4 on ILC1 and NCR- ILC3 populations in the SFMC mirrors previously published data describing reduced CCR4 expression on Th17 cells from the inflamed site (Nistala et al., 2008b). CCR6 has been previously shown to be expressed on synovial Th17 in JIA, where it is thought to play an important role in recruitment of these cells (NIstala 2008), (Nistala and Wedderburn, 2009, Cosmi et al., 2011). A trend towards higher CCR6 expression was seen in all synovial ILC subpopulations, suggesting that migration towards CCL20 could play a role in ILC recruitment to the joint. However, there was significant heterogeneity in CCL20 concentration in the SF. Therefore, a larger set of samples needs to be analysed to understand the heterogeneity of chemokine receptor expression on the ILC populations found in the JIA synovial fluid cells and how this relates to synovial fluid concentrations of specific chemokine ligands. It is possible that the concentration of ligands measure in aspirated synovial fluid may not reflect the microenvironment and local concentrations of chemokines or cytokines at the site of cell entry to the inflamed site across the endothelium

Taken together, these data support the hypothesis that chemokines at the inflamed site may contribute to ILC recruitment of ILC1 and ILC2. The high CCR4 and the increased CCR6 expression on ILC2 in the SFMC are particularly interesting since these results raise questions as to why the ILC2 subpopulation is reduced in SFMC. It is now well established that there is considerable plasticity among ILC subpopulations (Bernink et al., 2015, Silver et al., 2016b). Evidence of selective recruitment of ILC3 subpopulations is still weak and it is unclear whether the ILC3 population in the SFMC has migrated to the joint via CCR6 and other interactions, or whether this population has arisen from other ILC subpopulations, perhaps ILC2, at the inflamed site.

This plasticity hypothesis was subsequently investigated. Multiplex cytokine analysis of SF highlighted high levels of IL-1β, IL-6 and IL-15 in the synovial

fluid compared to paired blood and the influences of IL-1β and IL-6 were investigated further. Although IL-15 was not studied in this project, its effect on ILC activation and plasticity at the inflamed site would be interesting to investigate. It is known that ILC express the IL-2 receptor and respond to IL-2 stimulation, since IL-15 shares structural similarities to IL-2 and signals through a shared beta and gamma receptor chains. It is likely that ILC may also respond to IL-15 stimulation (Spits et al., 2013, Mishra et al., 2014). In fact, a recent study has shown that NK cells and ILC1 rely on IL-15 for their development, and proliferate and produce inflammatory mediators in response to stimulation (Dadi et al., 2016). Additionally, mouse studies have shown that IL-15 causes down regulation of RORγt and up regulation of Tbet in ILC3 (Vonarbourg et al., 2010, Klose et al., 2013). IL-15 could, therefore, be a significant influence on the ILC1 population within the inflamed joint in JIA.

It is interesting that IL-17A and associated cytokines, IL-22, IL-23 and GMCSF, were not elevated in synovial fluid samples, since previous studies have demonstrated high IL-17 levels in JIA patients (de Jager et al., 2007, Agarwal et al., 2008a). It is important to consider the limitations of the multiplex cytokine analysis used during this project, and associated issues with cytokine degradation after long-term storage. Previous studies have shown that while cytokines are broadly stable at -80°C for up to 2 years, after this time some cytokines are susceptible to degradation. Additionally, it has been shown that cytokines are susceptible to instability after freeze thawing (de Jager et al., 2009). It is therefore possible that the results obtained may represent an underestimation of the true cytokine abundance.

ILC plasticity was not conclusively demonstrated during the course of this project. One significant difficulty was the down regulation of ILC2 specific CRTH2 after *in vitro* culture. While it is possible that all ILC2 died during the cultures, or underwent phenotype switching, I cannot be sure that this was the case. This meant that I was unable to accurately assess the degree of phenotype switching, which may have occurred. The cell number after 7 days were too low to perform qPCR on the cultured ILC, which would help to clarify whether the ILC were still ILC2 or has switched to another ILC phenotype. In the

future, analysis of ILC subpopulation transcription factors before and after culture by PrimeFlow RNA assays could provide a more practical alternative.

The effects elicited by IL-1β and IL-23 alone or in combination with IL-6 remain unclear. Previous studies have shown that culture with IL-1β and IL-23 results in ILC switching from an ILC1 to an ILC3 phenotype (Bernink et al., 2015). An increase in cKit expression after culture with IL-6 was observed in the present study. The up regulation of cKit without induction of NKp44 could suggest that IL-6 promotes NCR-ILC3 survival, however, since ILC2 could not be identified after culture, there is insufficient evidence to definitively support this hypothesis. IL-6 is important for the differentiation of murine Th17 cells, although there are conflicting opinions regarding its influence on human T cells(Bettelli et al., 2006). Unfortunately, due to poor viability it was not possible to assess ILC cytokine production after culture, however one study in mouse and human IBD has suggested that IL-6 induces IL-17 production from human colonic ILC, which supports my hypothesis that IL-6 induces NCR-ILC3 and may be influencing this ILC subpopulation at the inflamed site(Powell et al., 2015). Further investigation of the role of IL-6 on ILC in the synovia is warranted.

Further optimization of the culture technique for ILC would be required to fully evaluate their plasticity in response to synovial cytokines. One major limitation of the assay stems from the small population size. Starting from $1x10^8$ aHC PBMC the average ILC yield after sorting was only approximately 20,000 ILC and cultures were set up with only 2000 ILC/ well. Therefore, a relatively small amount of death in some cultures meant that it was impossible to generate meaningful results at the end of culture due to insufficient cell numbers and data events collected during analysis. Other studies have cultured ILC with irradiated feeder cells to boost survival (Bernink et al., 2015, Lim et al., 2016). This method would definitely be worth exploring. Additionally, no other studies have attempted to culture bulk ILC, but instead work has focused on individually isolated ILC populations. I did not have sufficiently large samples available for this kind of analysis, however, isolating the individual ILC subpopulations would have allowed for more stringent analysis.

It is clear that there are cytokines enriched in the JIA synovia, which could promote ILC switching towards the ILC3 populations, however the source of these cytokines are still unknown (Bernink et al., 2015). Next, I focused on the presence of other immune cells in the SFMC that could be contributing to the cytokine milieu. A significant enrichment of mDC was characterized in the SFMC from JIA patients, confirming previously published data (Varsani et al., 2003). Although BDCA1+mDC, known to produce IL-6 and IL-23 following TLR-stimulation, were the predominant population, it is interesting that BDCA3+mDC were also dramatically enriched at the inflamed site. These cells are known to be efficient at cross presentation to CD8 T cells, and may identify one mechanism by which CD8 T cells are activated within the synovia(Dzionek et al., 2000, Jongbloed et al., 2010).

mDC correlated strongly with NCR+ILC3 within the SFMC, suggesting interaction between these two groups. Further interrogation of the mDC functionality by qPCR showed low gene expression of inflammatory cytokines, IL-1β, IL-23 and IL-6, cytokines implicated in ILC switching. Interestingly, synovial mDC had lower expression of these cytokine genes compared to aHC mDC. One explanation maybe that SFMC mDC exhibit an 'exhausted' phenotype. Previous studies have shown increased IL-10 at the inflamed site (Bending et al.,2015). It is possible that the mDC in the joint have succumbed to IL-10 mediated exhaustion (Kajino et al., 2007). However, it is more likely that synovial mDC may have entered a tolerogenic state and may be involved in promoting regulation at the inflamed site. In Chapter 4, I proposed a role for NCR+ILC3 in regulation at the inflamed site. The strong association of NCR+ILC3 with mDC at the SFMC may further support a tolerogenic role for synovial mDC. This hypothesis is strengthened by the association between mDC at the inflamed site and less severe disease according to Physician's VAS and ESR.

Further evidence in support of a tolerogenic nature for synovial mDC was the significantly greater CD103 expression seen on all DC subpopulations in the SFMC (compared to PBMC mDC). CD103 is associated with a population of mDC better able to produce RA, which has been established to promote Treg

differentiation and, more recently, with ILC phenotype switching towards NCR+ILC3(Coombes et al., 2007, Bernink et al., 2015). Additionally, RA has been shown attenuate intestinal inflammation by promoting binding of the retinoic acid receptor to the IL-22 promoter in γδ T cells. Indeed, RA mediates increased IL-22 and reduced IL-17 production from both γδ T cells and ILC3(Mielke et al., 2013). RA synthesis generally occurs in the gut, however it may also be induced in inflammatory conditions (Aoyama et al., 2013, Wang et al., 2014). Further functional investigation showed that synovial mDC expressed higher RALDH2 gene expression and higher ALDH activity compared to healthy mDC, suggesting an enhanced ability to make RA.

Finally, increased gene expression of RA receptor RARα on synovial ILC was also analysed by PCR. Expression was greater than that observed in CD161+ T cells, which are known to respond to RA(Duurland et al., 2017a). Collectively these data suggest that ILC may be able to respond to RA produced by mDC at the inflamed site. This hypothesis is supported by published data that demonstrated the ability of RA–producing DC to skew ILC towards NCR+ILC3, which are enriched in the JIA synovia, and which correlated with the mDC at the inflamed site (Bernink et al., 2015) Given the expression of RARα in synovial ILC, it was surprising that CCR9 expression was not also elevated on synovial ILC, since *CCR9* is a direct target of RA signalling (Ohoka et al., 2011). Although RA-dependent CCR9 expression has been shown previously in mouse ILC, the current data are in keeping with published studies showing that CCR9 is down regulated on synovial T cells (Ohoka et al., 2011, Kim et al., 2015, Duurland et al., 2017a). In T cells, RA induced CCR9 expression is depended on transient TCR signalling, but inhibited by aberrant stimulation. It may be the case that RA signalling in human ILC is insufficient to induce CCR9 expression. Alternatively, the synovial ILC may be highly activated and therefore unable to respond to RA signalling. Analyses in this Chapter were conducted on *ex vivo* ILC without stimulation. Short-term culture with RA could help to elucidate synovial ILC responses to RA and may show induction of CCR9. Additionally, further analysis of retinoid X receptor expression, and other targets downstream of RA signalling, such as integrin α4β7, could provide further insights into RA signalling in ILC at the inflamed site.

Taken together with previously published data, the results presented in this Chapter support a contribution of selective migration in ILC recruitment to the joint, and additionally provide evidence that cytokines in the synovial fluid including IL-6 may influence ILC phenotype. Finally, this Chapter suggests a role for mDC-derived RA in ILC phenotype switching at the inflamed site.

6 : Final Discussion

This thesis presents novel data describing the IL-17 signature in JIA, and more specifically ERA JIA. Analysis of paired PBMC and SFMC patient samples has enabled valuable insight into immune pathology at the site of inflammation. In addition to exploring T cell derived IL-17 this thesis explores the putative role of a newly described immune cell population, ILC, in pathogenesis of JIA. Finally, steps have been taken to elucidate the mechanisms that underpin a subpopulation-specific ILC expansion within the inflamed synovia.

6.1 IL-17 – a marker for stratified treatment in JIA?

There is a universal drive towards stratified medicine in decision making to ensure optimal and efficient treatment of disease (Arron et al., 2015). There are a number of biological therapies available for the treatment of JIA, targeting key cytokines believed to be fundamental in driving and maintaining disease pathogenesis. Current treatments are predominantly aimed at targeting TNFα and more recently IL-6: however these new medications are useful only in a proportion of patients (Lovell et al., 2006, Klein and Horneff, 2009). There remains a group of patients who are unresponsive to these treatments and therefore there is a continued need for improved, specific therapies. New treatments targeting the IL-23/IL-17 axis are now gaining momentum for the treatment of JIA after proving effective for the treatment of adult arthropathies (Chiricozzi and Krueger, 2013, McInnes et al., 2014, Sofen et al., 2014, Poddubnyy et al., 2014, Baeten et al., 2015).

IL-17 has long been implicated in the pathogenesis of murine and human arthropathies. IL-17 mediates disease by promoting neutrophil influx by inducing secretion of chemo-attractants and by inducing cartilage and bone destruction in combination with TNFα and IL-1β (Chabaud et al., 2001, Ye et al., 2001, Murphy et al., 2003). It also induces production of further proinflammatory molecules including both cytokines (TNF, IL-1, IL-6, GM-CSF) and chemokines (CXCL2, CXCL8, CCL20)(Chabaud et al., 2001, Hwang et al., 2004, Liang et al., 2006). It is well established that there is a predominance of Th17 cells in the inflamed joints of patients with oligo- and poly-JIA, however little has been

previously described of this signature in ERA JIA (Nistala et al., 2008a, Agarwal et al., 2008b). The IL-23/IL-17 axis is implicated, in part, due to the close association of ERA JIA with the HLA-B27 allele commonly associated with IL-17 mediated disease (Edmonds et al., 1974). Further hypotheses are based on the genetic and clinical similarities between ERA-JIA and adult AS, in which Th17 cells are reported to drive disease. Both ERA JIA and AS also share a genetic association with the locus ERAP1 which codes for endoplasmic reticulum aminopeptidase 1, which is important for the optimization of peptides for MHC presentation (Saric et al., 2002, Hinks et al., 2011, Evans et al., 2011). Finally, GWAS studies have identified disease associated SNP in the *IL23R* gene in JIA (Emami et al., 2016)(Hinks et al., 2011). However, cellular analysis in ERA JIA to date has been predominately focused on innate immune cells including monocytes, and has been largely restricted to Indian cohorts (Saxena et al., 2005, Newport et al., 2007, Zhou et al., 2007, McGeachy et al., 2009, Mahendra et al., 2009, Myles and Aggarwal, 2011, Cortes et al., 2013, Rahman et al., 2014).

This thesis presents data demonstrating a Th17 enrichment in the joints of ERA patients from a UK cohort, above what has been previously reported in other subtypes of JIA (Nistala et al., 2008a). Additionally, these data demonstrate that the IL-17 signature extends beyond the CD4 T cell compartment as it is also observed in CD8 and γδ T cells. Importantly, IL-17+ T cells demonstrate a positive association with measures of disease severity supporting a role for these cells in promoting pathogenesis. It is interesting to note that the results described in this thesis echo what has been described in other HLA-B27-associated adult arthropathies including AS (Shen et al., 2009, Bowness et al., 2011, Jansen et al., 2015) (Gracey et al., 2016). These immunological parallels suggest that the similarities between ERA JIA and AS extend beyond clinical symptoms or genetics, but to potential pathological mechanism(s) at the inflamed site.

When assessing the contribution of IL-17 to disease pathogenesis it is important to consider IL-17 production by immune cells other than T cells. In parallel to the T cell data described, this thesis shows a significant enrichment of NCR-

ILC3 within the synovial fluid from JIA patients compared to paired blood. These cells are known to be potent producers of IL-17 (Gladiator et al., 2013, Hazenberg and Spits, 2014a) and are shown here to be correlate with more severe disease. These results mirror what has previously been reported in other human studies of inflammatory diseases including adult psoriasis, psoriatic arthritis, IBD and AS (Geremia et al., 2011, Teunissen et al., 2014a, Ciccia et al., 2014b, Leijten et al., 2015, Yeremenko et al., 2015). The analyses presented here do not show a significant difference in ILC subpopulation proportions between JIA subtypes. These observations need confirmation. One potential explanation of the current data may be the limited number of patient samples investigated in this study and it is likely that significant differences may emerge with an extended study. Indeed, when the proportion of synovial Th17 cells within the CD4+ T cell population were analysed in relation to NCR-ILC3 frequency, a strong positive correlation was seen. Since it has been shown that Th17 cells are highest in the ERA subtype of JIA, it is likely that the NCR-ILC3 are also most prevalent in this clinical subtype. That said, there is a minority of patients of other JIA subtypes who also present a strong IL-17 signature. It is important to consider that the ILAR JIA subtypes do not represent strict diagnoses. There are significant clinical overlaps between subtypes. It is possible that some patients with other JIA subtypes may even carry the HLA-B27 allele.

Together, my findings identify a subgroup of ERA JIA/ JIA patients with an IL-17 predominance. In these patients IL-17-producing cells are associated with more severe disease, supporting a role for IL-17 in disease pathogenesis. This subgroup of patients should be ideal candidates for therapies targeting the IL-23/IL-17 pathway. Future work should focus on the development of a minimally invasive and robust strategy for the identification of these patients in the clinic. It would be very valuable to identify a specific biomarker for those patients with a strong synovial IL-17 signature so that a stratified approach to treatment could be adopted. There is recently increased interest in the search for biomarkers for the stratification of JIA patients to ensure optimal treatment. The ideal biomarker should be detectible in biological media, ideally which can be obtained with minimal discomfort to the patient, such as blood or urine.

Additionally, it is important for a biomarker for clinical use to be stable molecules which will not degrade rapidly at room temperature. Finally, it is crucial for the tests utilised for the detection of biomarkers to be affordable, reliable and simple to carry out in the clinic.

The T cell and ILC analyses presented did not consistently find significant enrichments of IL-17 producing cells in the peripheral blood of those patients who demonstrated expanded IL-17+ populations within the synovial fluid. Interestingly, a recent study in JIA has suggested that a 'pathogenic' CD4 T cell phenotype can be identified in the peripheral blood of patients with JIA with the same TCRβ-chain CDR3 clones identified in the joint. In this study, these so called "circulating pathogenic-like lymphocytes", CPLs, made more proinflammatory cytokines in an *in vitro* assay including IL-17, compared to other CD4 T cells from the same patient (Spreafico et al., 2016). This suggests that analysis of T cell clones may identify pathogenic T cells in the peripheral blood of patients; this emerging information should be confirmed in future studies.

ILC3 cells can be divided into two distinct populations, depending on their expression of the NCR receptor (Hazenberg and Spits, 2014b). Adding complexity to the data reported here, I observed increased frequency of NCR+ILC3 at the inflamed site in JIA. These cells produce IL-22 and other cytokines, (Cella et al., 2009, Ren et al., 2011). The data presented here suggest that in the context of JIA these cells appear to be present in a reciprocal relationship to their IL-17-producing counterparts, NCR-ILC3 cells. While NCR-ILC3 are associated with more severe disease, NCR+ILC correlate with improved patient outcomes. This is surprising since IL-22-producing ILC3 have been shown to exacerbate inflammation in adult rheumatoid arthritis. In the context of rheumatoid arthritis, ILC-derived IL-22 and TNFα induce proliferation of synovial fibroblasts (Ren et al., 2011). However, the identification strategy employed during this study diverges from the strategy utilized in this thesis making a direct comparison difficult, highlighting that these conclusions should be treated with caution(Spits et al., 2013). More recently, it has been reported that IL-22-producing ILC3 can inhibit inflammatory IL-17+ T cell

responses via AHR signalling in murine models of IBD, supporting a protective role for NCR+ILC3 (Qiu et al., 2013).

Since both NCR-ILC3 and NCR+ILC3 require IL-23 signalling for their development and function, it is important to take into account the putative protective role for NCR+ILC3 when considering treatment options(Geremia et al., 2011, Hazenberg and Spits, 2014a). Treatments targeting IL-23, such as Ustekinumab or Guselkumab, may not yield optimal outcomes, as their effects are likely to cause blockade of the NCR+ILC3 population. In this context direct targeting of downstream inflammatory IL-17 may prove more prudent.

Since the precise contribution of these ILC subpopulations to JIA pathogenesis (or resolution of disease) still remains elusive, humanised animal models of arthritis could provide useful insights and a unique prospect to answer these questions.

6.2 Does the JIA synovial environment drive ILC phenotype switching?

Data presented in Chapter 5 shows increased expression of chemokine receptors, CXCR3, CCR4, CCR5 and CCR6, on synovial ILC supporting a contribution of chemokine-mediated migration of ILC to the joint in JIA. However, selective migration does not completely explain the skewed ILC phenotype reported in the SFMC of patients with JIA. Understanding how immune cells function and change within the inflamed site is crucial to understanding the mechanisms that underpin disease. A good example of this in the context of JIA is the reported plasticity between Th1 and Th17 cells within the joints of patients. In this context, T cell plasticity likely results in more severe disease. It is suggested that Th1-derived IFNγ may induce secretion of CCL20 from APC (Kryczek et al., 2008). This CCL20 subsequently recruits Th17 cells to the inflamed site where they perform their functions. In a positive feedback loop, synovial IL-12 may then promote phenotype switching of the recruited Th17 cells to IFNγ-producing T cells, maintaining pathology(Nistala et al.,

2010a). These switched “ex-Th17” Th1 cells still produce cytokines, not only IFNγ but also for example GM-CSF (Piper et al., 2014b)

ILC mirror T cell subsets in their cytokine profiles and rely on similar differentiation signals to their T cell counterparts(Hazenberg and Spits, 2014a). There is now evidence for considerable plasticity between ILC subpopulations. Most of this work has been conducted in mouse models and has predominantly focused on plasticity between ILC1 and ILC3 populations. In parallel to T cells, IL-12 is significant for maintaining an ILC1 phenotype, while IL-23 and IL-1β play a role in promoting ILC3 populations (Nistala et al., 2010b, Cella et al., 2010, Piper et al., 2014b, Bernink et al., 2015, Lim et al., 2016).

This thesis presents evidence for the first time that ILC phenotype switching may occur at the inflamed site in JIA, although at this stage only tentative conclusions can be drawn. Multiplex cytokine analysis revealed an abundance of inflammatory cytokines within the synovial fluid, which may play a role in promoting ILC phenotype plasticity. Of particular interest is the high concentration of IL-6 within the inflamed synovia. IL-6 has previously been implicated in both Th17 development and activation of IL-17-producing ILC3 (Bettelli et al., 2006) (Powell et al., 2015). Future work should be aimed at dissecting the pleiotropic effects of IL-6 on ILC3 biology *ex-vivo* and *in vivo*.

During this study, total ILC were sorted by flow cytometry from healthy PBMC and cultured. Unfortunately, the ability to draw firm conclusions from these experiments, presented in Chapter 5, was hampered by the fact that ILCs rapidly down-regulated some of the subpopulation-specific surface markers, including CRTH2, crucial for the identification of ILC subpopulations. It is suggested that IL-4 is required for the maintenance of CRTH2 and ILC2 function *in vitro* (Bal et al., 2016, Silver et al., 2016b). However, without further analysis it is still unclear whether ILC, which have down regulated CRTH2, have undergone phenotype switching, or remain functionally active. Previously published studies have cultured purified ILC subpopulations isolated from human and mouse tissues in order to assess mechanisms of plasticity (Bernink et al., 2015, Bal et al., 2016). Due to the small size of the ILC population in

human blood, this was not possible in the present study. Other studies have utilized ILC cloning techniques to follow ILC phenotype changes (Bernink et al., 2015). Although technically very challenging, I believe this represents the best option to reveal the effects of synovial cytokines on ILC plasticity.

In addition to IL-23 and IL-1β, a recent study has identified an important role for retinoic acid (RA) in augmenting an ILC3 phenotype (Bernink et al., 2015). In data presented in Chapter 5, an enrichment of mDC within the SFMC from JIA patients capable of producing RA was recorded, in addition increased expression of RA receptor gene *RARA* on synovial ILC was also observed. This clear enrichment of RALDH-producing mDC was demonstrated across many subtypes of JIA although due to small numbers of patients analysed in some subtypes these were not analysed separately. Together, my findings support a role for synovial RA in ILC skewing in JIA. However, further validation is required. It is interesting that CCR9 expression was not elevated on synovial ILC since the CCR9 gene is directly downstream of RA signalling (Ohoka et al., 2011). This observation aligns with recently published data from the Wedderburn group showing reduced CCR9 expression on synovial T cells (Duurland et al., 2017a). Whether this CCR9 down regulation is indicative of impaired RA signalling remains unclear. Further analysis of RA-responsive genes may shed light on this.

A recent study has highlighted a pivotal role for IL-15 in the maintenance of pathogenic Th17 cells in autoimmunity (Chen et al., 2017). I have demonstrated significant amounts of IL-15 in the synovial fluid from JIA patients, although the role of this cytokine in ILC plasticity was not investigated. ILC are known to express the IL-2 receptor, which shares beta and gamma chains with the IL-15 receptor (Hazenberg and Spits, 2014b, Mishra et al., 2014). Interestingly, recent murine studies have identified a role for IL-15 in the development and activation of ILC1, further the cytokine causes down regulation of RORγt in ILC3 (Vonarbourg et al., 2010, Klose et al., 2013, Dadi et al., 2016). I hypothesize that this mechanism translates to the human setting, and IL-15 drives ILC3/ILC1 plasticity within the inflamed joints of JIA patients.

6.3 Future directions

This thesis and previous research by the Wedderburn group has identified an expansion of GM-SCF+ T cells at the inflamed site in JIA (Piper et al., 2014b). ILC-derived GM-CSF was not examined during the course of this study. However, given the parallels seen between T cells and ILC within the synovial fluid, it is tempting to speculate that synovial ILC produce significant GM-CSF. The potential effect and role of GM-CSF warrants further investigation for several reasons:

Firstly, GM-CSF is a significant player driving inflammation in mouse models of disease and is also implicated in pathogenesis of adult forms of arthritis (Cook et al., 2011, Codarri et al., 2011). GM-CSF has been shown to maintain disease in mouse models of MS. This is the case even in the absence of IL-17, where GM-CSF activates other immune cells causing aberrant inflammation and tissue damage. Furthermore, GM-CSF-producing T cells are heavily implicated in the pathogenesis of adult rheumatoid arthritis and new clinical trials targeting GM-CSF and its receptor have shown efficacy and safety in phase two trials (Behrens et al. 2014; Burmester et al. 2013). A putative role for GM-CSF in driving pathogenesis of JIA opens the door for novel therapeutics and further stratification of patients.

Secondly, in contrast to its inflammatory role, GM-CSF, and in particular IL-22+ILC3-derived GM-CSF has recently been shown to promote RA production from mDC (Magri et al., 2014). The work presented in this thesis in addition to previous publications describes a potential role for RA in skewing ILC towards potentially protective NCR+ILC3 (Bernink et al., 2015). Considering these findings together, raises a hypothesis that a positive feedback loop in the inflamed joint in which NCR+ILC3 produce GM-CSF, which then induces mDC to produce RA, which in turn supports the differentiation of NCR+ILC3 (Magri et al., 2014). If this is the case and NCR+ILC3 are shown to be protective in JIA, treatment decisions should take into account the constructive role of GM-CSF, particularly in those patients in which the NCR+ILC3 population is highly

enriched. In these cases, targeting molecules further down-stream such as IL-17 may make more effective targets.

The developmental and functional similarities between NK cells and CD127+ILC highlights areas for further investigation, both in the context of ILC biology and in terms of HLA-B27-mediated disease. One hypothesis for the pathogenesis of HLA-B27 is its ability to form heavy chain homodimers (Allen et al., 1999, Kollnberger et al., 2004, Colbert et al., 2014). These homodimers may be expressed on the surface of cells or within the cytoplasm and can be detected by NK cell KIRs(Kollnberger et al., 2002, Kollnberger et al., 2007, Cauli et al., 2013). Ligation of HLA-B27 dimers with KIRs is shown to induce an IL-17 phenotype in NK cells and T cells (Bowness et al., 2011, Ridley et al., 2016). It is known that CD127+ILC may express markers typically associated with NK cells. Indeed ILC3 populations are defined by expression of NK triggering receptor NKp44(Hazenberg and Spits, 2014b). The expression of KIRs has not been investigated in CD127+ILC populations to date. However since ILC, NK cells and T cells arise from a common progenitor cell it is possible that ILC may also express KIRs. This may represent one mechanism by which IL-17-producing ILC are activated in ERA JIA. Previous studies have investigated the expression of KIR-3DL1 and KIR-3DL2 on T cells and NK cells. Analysis of these receptors on CD127+ILC should be conducted and, if expressed, the impact of ILC KIR binding to B27 homodimers could be assessed by culture techniques.

This thesis confirms that the T cells populating the synovial fluid are predominantly of a memory phenotype and thus functionally different to their peripheral counterparts(Silverman et al., 1993, Wedderburn et al., 2000a, Chiesa et al., 2004). Recently, the concept of ‘trained immunity’ in innate cells has been coined and alludes to a memory type function in prototypic innate immune cells(Netea et al., 2011). This idea was initially described in NK cells (Cooper et al., 2009, Berrien-Elliott et al., 2015). Several studies have now identified populations of long-lived NK cells in vaccinated individuals with heightened cytokine responses (Sun et al., 2009a, White et al., 2014). These ‘memory’ NK cells respond quickly after re-stimulation to produce increased

levels of IFNγ and are implicated in pathogenesis of allergic disease(O'Leary et al., 2006). Various mechanisms for the development of trained immunity have been postulated, although a lot remains to be uncovered. It is known that the interaction of innate immune cells with their target PAMPS results in profound changes in expression patterns of surface receptors on these cells (Bowdish et al., 2007, Sun et al., 2009b). In addition, chromatin modulation and accessibility, modulated by histone methylation and acetylation are important for inducing memory in innate immune cells (Foster et al., 2007, Carson et al., 2011). Although technically very challenging, analysis of PRR expression and epigenetics of ILC from inflamed sites could provide exciting insight into functionality of these cells and further clarify their role in promoting pathogenesis of inflammatory disease.

6.4 Concluding remarks

Despite rapid advancements in treatment strategies for childhood arthritis, there are still a proportion of patients who fail to achieve clinical remission. While the pathogenesis of several subtypes of JIA has been well examined, there is little known about the pathogenesis of ERA JIA. Previous studies into pathogenesis of JIA have typically focused on the cytokine contribution of CD4 T cells. This thesis marks the first report of ILC in childhood arthritis, and takes steps to elucidate the mechanisms by which these cells populate the inflamed synovia. The present study clearly demonstrated a heightened IL-17 signature across T cell subtypes and ILC in the synovial fluid from ERA patients. Additionally, I have identified a subgroup of JIA patients who make ideal candidates for novel treatments targeting the IL-23/IL-17 axis. Further analysis of synovial ILC populations has highlighted the importance of careful consideration when making treatment decisions targeting the IL-23/IL-17 pathway to ensure that only pathogenic cells are targeted. In addition, several cytokines and immune mediators within the synovial fluid, including IL-6, IL-15 and RA, identified may be significant for ILC plasticity and function at the inflamed site. Finally, several novel hypotheses are presented for future investigation into ILC memory and the pathogenic roles for ILC in JIA.

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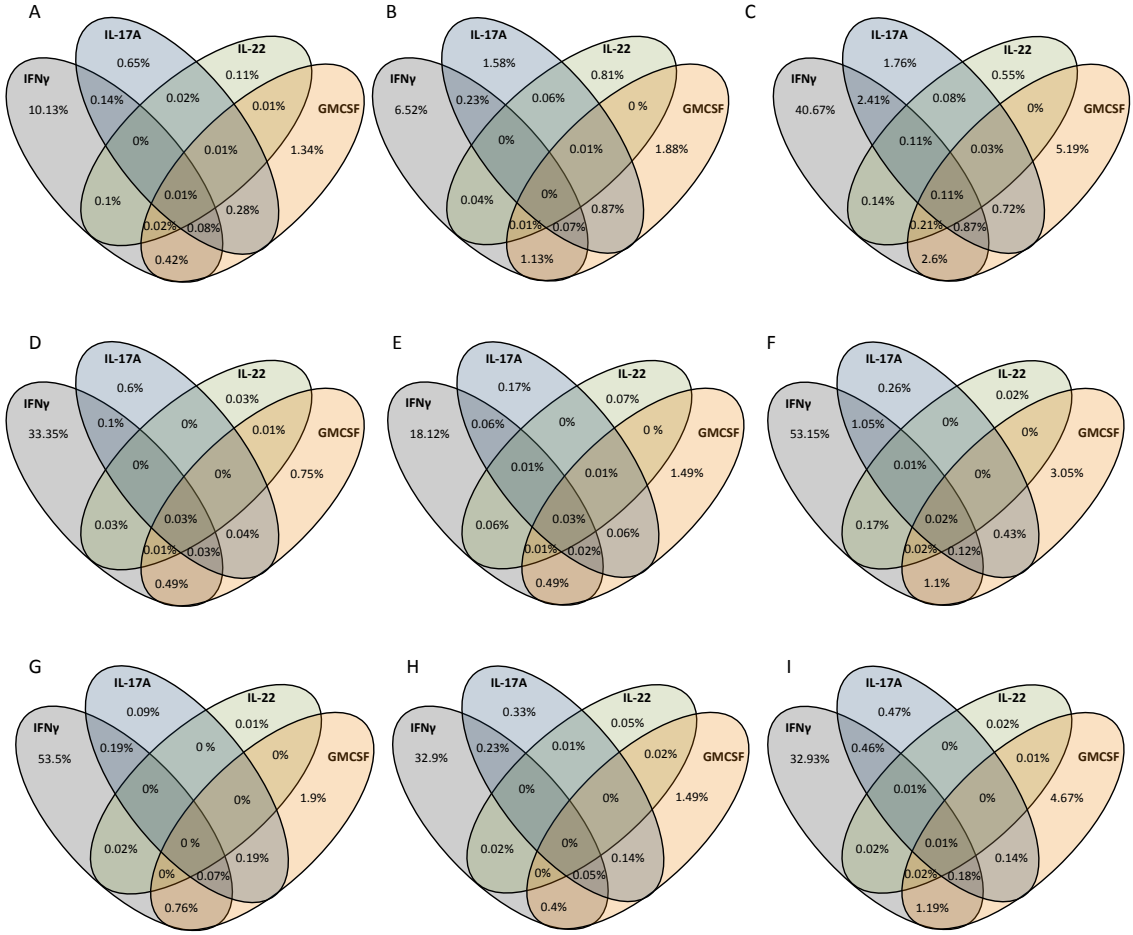
Appendices



Appendix 1. Polyfunctional T cells are expanded in SFMC from ERA JIA patients.

Summary Venn diagrams showing mean cytokine positive cells and coproduction for IFNγ, IL-17A, IL-22 and GM-CSF from PBMC from aHC (n=4), and PBMC (n=6) and SFMC (n=6) from ERA JIA patients. (A-C) aHC (A) CD4 T cells; (B) CD8 T cells and (C) CD4-CD8- T cells. (D-F) ERA JIA PBMC (D) CD4 T cells; (E) CD8 T cells and (F) CD4-CD8- T cells. (G-I) ERA JIA SFMC (G) CD4 T cells; (H) CD8 T cells and (I) CD4-CD8- T cells.

List of publications arising from this work or contributed to during this PhD programme

1. BENDING, D., PESENACKER, A. M., URSU, S., WU, Q., **LOM, H**., THIRUGNANABALAN, B. & WEDDERBURN, L. R. 2014. Hypomethylation at the regulatory T cell-specific demethylated region in CD25hi T cells is decoupled from FOXP3 expression at the inflamed site in childhood arthritis. *J Immunol,* 193**,** 2699-708.
2. BENDING, D., GIANNAKOPOULOU, E., **LOM, H**. & WEDDERBURN, L. R. 2015. Synovial Regulatory T Cells Occupy a Discrete TCR Niche in Human Arthritis and Require Local Signals To Stabilize FOXP3 Protein Expression. *J Immunol,* 195**,** 5616-24.